Department of Pathology
Research Program of Molecular Neurology
University of Helsinki
Finland

Interactions and turnover of the muscular dystrophy protein myotilin

Pernilla von Nandelstadh

ACADEMIC DISSERTATION

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SUPERVISED BY
Professor Olli Carpén, M.D., Ph.D.
Department of Pathology
University of Turku, Finland
Department of Pathology
University of Helsinki, Finland

REVIEWED BY
Professor Pekka Lappalainen, Ph.D.
Institute of Biotechnology
University of Helsinki, Finland

Docent Katarina Pelin, Ph.D.
Department of Biological and Environmental Sciences
University of Helsinki, Finland

OPPONENT
Professor Jari Ylänne, Ph.D.
Department of Biological and Environmental Sciences
University of Jyväskylä, Finland

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Yliopistopaino
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To my family

Contents

Abbreviations

ABP	Actin binding protein
AD	Autosomal dominant
AR	Autosomal recessive
ALP	Alpha-actinin associated LIM protein
Amp	Ampicillin
BMD	Becker's muscular dystrophy
CaM Kinase	Ca2+/calmodulin-dependent protein kinase
CHCA	α-cyano-4-hydroxy cinnamic acid
CLP36	36 kDa C-terminal LIM domain protein
Cm	Chloramphenicol
C-terminus	Carboxy terminus
DCM	Dilated cardiomyopathy
DGC	Dystroglycan complex
DMD	Duchenne muscular dystrophy
ECM	Extracellular matrix
F-actin	Filamentous actin
Fn	Fibronectin
FKRP	Fukutin-related protein
G-actin	Globular actin
GFP	Green fluorescence protein
GST	Glutathione S-transferase
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilodalton(s)
LIM	Lin-11, Isl1 and Mec-3
LGMD	Limb-girdle muscular dystrophy
MD	Muscular dystrophy
MG132	Z-Leu-Leu-Leu-al
MFM	Myofibrillar myopathy
MS	Mass spectrometry
MyBP-C	Myosin binding protein C
Myotilin	Myofibrillar titin-like protein
N-terminus	Amino terminus
PDZ	Postsynaptic density 95, discs large and zonula occludens-1
PKA	Protein kinase A
PKC	Protein kinase C
PR	Proline-rich
RT	Room temperature
SBM	Spheroid body myopathy
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tm	Tropomyosin

Tn	Troponin
Trim 32	Tripartite motif-containing 32
UPS	Ubiquitin-proteosom system
Wt	Wild type
ZASP	Z band alternately spliced PDZ-containing protein
Z-LLal	Z-Leu-Leu-H

Original publications

This thesis is based on the following publications:

I **von Nandelstadh, P**., Gronholm, M., Moza, M., Lamberg, A., Savilahti, H. & Carpen, O. 2005, "Actin-organising properties of the muscular dystrophy protein myotilin", *Experimental cell research*, vol. 310, no. 1, pp. 131-139.

II **von Nandelstadh, P**.*, Ismail, M.*, Gardin, C., Suila, H., Zara, I., Belgrano, A., Valle, G., Carpen, O. & Faulkner, G. 2009, "A class III PDZ binding motif in the myotilin and FATZ families binds enigma family proteins: a common link for Z-disc myopathies", *Molecular and cellular biology*, vol. 29, no. 3, pp. 822-834.

III **von Nandelstadh, P**., Solyimani, R., Baumann, M. & Carpen, O. "Analysis of myotilin turnover provides mechanistic insight on the role of myotilinopathy-causing mutations", *Submitted*.

* equal contribution

The publications are referred to in the text by their roman numerals.

Abstract

The striated muscle sarcomere is a force generating and transducing unit as well as an important sensor of extracellular cues and a coordinator of cellular signals resulting in various adaptive responses. As an example, mechanical signals (e.g. stretch) are sensed at the sarcomeric Z-disk and converted to biochemical events and changes in transcriptional activity. Myotilin, a Z-disk component identified by us, interacts with Z-disk core structural proteins and with regulators of signaling cascades and MuRF ubiquitin ligases. Missense mutations in the gene encoding myotilin cause two types of dominantly inherited disorders, myofibrillar myopathy (MFM) and limb-girdle muscular dystrophy 1A (LGMD1A) as well as cardiomyopathy by an unknown mechanism.

In this thesis, consisting of three publications, the functions of myotilin were further characterized to clarify the molecular biological basis and the pathogenetic mechanisms of inherited muscle disorders, mainly LGMD1A, MFM, and cardiomyopathy caused by mutated myotilin.

Myotilin has an important function in the assembly and maintenance of the Z-disks probably through its actin-organizing properties. We used a number of truncated and mutated myotilin variants and several cell biological and biochemical methods, including transposon mutagenesis and yeast two-hybrid method, to further dissect its unique actin binding and bundling functions. Our results show that the Ig-domains of myotilin are needed for both binding and bundling actin and define the Ig domains as actin-binding modules. The disease-causing mutations appear not to change the interplay between actin and myotilin.

Interactions between Z-disk proteins regulate muscle functions and disruption of these interactions results in muscle disorders. Mutations in Z-disk components myotilin, ZASP/Cypher and FATZ-2 (calsarcin-1/myozenin-2) are associated with myopathies. We used various biochemical binding assays, including AlphaScreen and PDZ array membranes, bioinformatics, microscopy and phosphorylation experiments to study the potential interplay between myotilin, ZASP and FATZ-2. We showed that proteins from the myotilin and FATZ (calsarcin/myozenin) families interact via a novel and unique type of class III PDZ binding motif with the PDZ domains of ZASP/Cypher and other Enigma family members and that the interactions can be modulated by phosphorylation.

The morphological findings typical of myotilinopathies include Z-disk alterations and aggregation of dense filamentous material. The causes and mechanisms of protein aggregation in myotilinopathy patients are unknown, but it has been suggested that impaired degradation might explain in part the abnormal protein accumulation. We explored, whether myotilin is degraded by the calcium-dependent, non-lysosomal cysteine protease calpain and by the proteasome pathway, and whether wild type and mutant myotilin differ in their sensitivity to degradation. We showed that myotilin is a substrate for calpain and mapped two of the calpain cleavage sites by mass spectrometry. These studies identify the first functional difference between mutated and wild type myotilin. Furthermore, if degradation of myotilin is disturbed, it accumulates in cells in a manner resembling that seen in myotilinopathy patients. Based on the results, we propose a model

where mutant myotilin escapes proteolytic breakdown and forms protein aggregates, leading to disruption of myofibrils and muscular dystrophy.

In conclusion, the main results of this study demonstrate that myotilin is a Z-disk structural protein interacting with several Z-disk components. The turnover of myotilin is regulated by calpain and the ubiquitin proteasome system and mutations in myotilin seem to affect the degradation of myotilin, leading to protein accumulations in cells. These findings are important for understanding myotilin-linked muscle diseases and designing treatments for these disorders.

Review of the literature

1. The cytoskeleton

Eukaryotic cells have internal scaffolding called the cytoskeleton that gives them their distinctive shapes. It also enables the cell to move or the muscle cell to contract and is required for cell division and transport of organelles inside the cell.

The cytoskeleton is an organized network of three different, but interconnected filament structures: microtubules, the intermediate filaments, and microfilaments. The cytoskeleton is not a static structure, as its name implies, but cytoskeletal polymers are highly dynamic, capable of polymerizing, depolymerizing, and moving within the cytoplasm on a time scale of seconds to minutes. All three types of filaments form as helical assemblies of subunits that self-associate using a combination of end-to-end and side-to-side protein contacts. The three cytoskeletal systems are interconnected via proteins that are able to bind the different cytoskeletal proteins. For instance, during cell division and cell migration, microtubule and actin cytoskeletons need to perform their tasks in an orderly manner (reviewed in Frixione, 2000).

Microtubules are strong, rigid hollow tubes. They function in organizing the cytoplasm and transporting organelles like vesicles or mitochondria within the cytoplasm. In intracellular trafficking, the vesicles glide along the microtubules with the help of motor proteins (such as kinesin and dynein) to their targets. During cell division, a large dynamic array of microtubules, the mitotic spindle, functions to physically segregate the chromosomes and to orient the plane of cell cleavage. Microtubules are also involved in neurite outgrowth and the movement of flagella and cilia (reviewed in Gardner et al., 2008).

Intermediate filaments are rope-like protein fibers that tolerate stretching and bending and are hard to break. The nuclear lamins form a network of filaments on the inner surface of the nuclei and create a structural scaffold for the nuclear envelop. The cytoplasmic intermediate filaments are not required in every cell type and these intermediate filaments are very diverse. Keratin filaments in epithelial cells form skin, nails and hair and neurofilaments provide mechanical strength in nerve cells and desmin filamnets in muscle cells (reviewed in Chang & Goldman, 2004).

Many debilitating human diseases, including cancer, developmental diseases, and neurodegenerative diseases, are linked to defects in the cytoskeleton (Lundin et al., 2010).

1.2 The actin cytoskeleton

Actin is the most abundant intracellular protein in a eukaryotic cell. In muscle cells, for example, actin comprises 10 % by weight of the total cell protein and in nonmuscle cells, up to 5 % of the cellular protein is actin. The 42 kDa actin monomer is encoded by a large, highly conserved gene family. The amount of actin genes varies from one in some single-celled eukaryotes like yeasts and amebas to several in multicellular organisms. For

instance, humans have six actin isoforms coded by separate genes, which are divided into three groups: alpha (α), beta (β), and gamma (γ) and some plants have as many as 60 actin isoforms (Khaitlina, 2001).

The most variable region in the actin molecule is the N-terminal end. Although the isoforms differ at only four or five positions, they have different functions. In vertebrates, α-actins are expressed mainly in muscle cells (α-skeletal, α-cardiac, α-smooth muscle, and γ- smooth muscle). In human skeletal muscles, α-skeletal actin is the predominant isoform while α -cardiac actin is the most abundant isoform in the heart tissue. Furthermore, α -smooth muscle actin is the major isoform in vascular tissues such as the aorta, while γ-smooth muscle actin predominates in the gastrointestinal and genital tracks. β- and γ-isoforms are found in non-muscle cells (β- and γ1-cytoplasmic) and are ubiquitously expressed (Khaitlina, 2001).

Actin exists as globular monomers called G-actin and as linear chains of G-actin subunits forming filamentous polymers called F-actin. These are about 8 nm in diameter and, being the thinnest of the cytoskeletal filaments, are also called microfilaments (or thin filaments in skeletal muscle fibers). The ability of G-actin to polymerize into F-actin and of F-actin to depolymerize into G-actin is an important property of actin and vital for several key cellular events such as cell motility, cell division, and endocytosis. Rapid polymerization and depolymerization of actin filaments occurs via binding and hydrolysis of ATP. Free actin monomers bind ATP and are incorporated onto the fast growing barbed end of the filament. ATP is then hydrolyzed to ADP and Pi. Dissociation of ADP-actin at the opposite pointed end causes disassembly of the filament. Actin treadmilling occurs when the association rate of free ATP-G-actin to the ends of actin filaments is balanced by the rate of subunit loss and no net growth occurs. Actin treadmilling is powered by ATP hydroylsis and this energy can be used to perform work (Pollard et al., 2000).

The length of actin filaments is controlled by actin binding proteins (ABP). Capping proteins prevent assembly at the barbed end while ADF/cofilin binds to the side of ADP-actin filaments to cause disassembly of the filament. In the absence of actin-binding proteins, the filament length is stable by the treadmilling mechanism. Profilin enhances filament assembly by promoting ADP to ATP exchange on actin and by directing actin monomers to the barbed end of filaments (Le Clainche et al., 2008). The Arp2/3 complex is involved in the organization of the actin network and it binds to the sides of existing filaments and initiates growth (nucleates) of new filaments creating a branched actin network (Small et al., 2002).

In multicellular organisms, the actin cytoskeleton is required for several morphogenetic processes, such as movement of neurites during development, remodeling of the nervous system, and chemotactic movements of for example fibroblasts during wound healing. In muscle tissue, actin filaments participate in muscle contraction. Actin filaments are concentrated under the plasma membrane, where they form various structures that help cells to move. These protrusive structures of the plasma membrane are called lamellipodia or leading edge, filopodia, and pseudopodia. All of these structures contain different, specialized actin networks, depending on the accessory proteins participating in network formation (Pollard et al., 2000).

Stress fibers are thick bundles of approximately 10-30 actin filaments traversing the cell (reviewed in Pellegrin et al., 2007, Naumanen et al., 2008). These bundles are held together by the actin-crosslinking protein α-actinin, although other actin-bundling proteins, such as fascin, espin and filamin, have also been detected in these regularly spaced thickenings called dense bodies. In addition to actin cross-linking proteins, the dense bodies are composed of scaffolding proteins and probably also transiently of proteins involved in signaling, such as kinases. The staining pattern of α-actinin is periodic along the fiber and alternates with bands containing non-muscle myosin and tropomyosin. The stress fibers have the ability to contract and via adhesions transmit the generated energy to the extracellular matrix (ECM) and are thus suggested to resemble the sarcomeric actin filament structures of muscle cells. The contractile force of the stress fibers depends on the cellular needs. Tissue fibroblasts have sparse and poorly organized contractile actomyosin bundles, whereas smooth muscle cells are highly contractile cells with highly organized actomyosin arrays (Pellegrin et al., 2007).

Stress fibers link the cell interior to the exterior through focal adhesions. Mammalian cells contain three categories of stress fibers: ventral stress fibers that are attached to focal adhesions at both ends, dorsal stress fibers that are attached to focal adhesions typically at one end and transverse arcs that are curved acto-myosin bundles, which do not directly attach to focal adhesions. Imaging of stress fiber formation in living cells shows that each type of stress fiber is assembled by a different mechanism (Hotulainen & Lappalainen, 2006).

The stress fibers are formed when physical stress is applied to the cells. Under normal conditions the three-dimensional ECM protects *in vivo* most cells against such forces, and only a few cell types including endothelial cells are under constant direct physical stress. When the support of the ECM is compromised, by a wound in the dermis for instance, the cells react rapidly to this new situation by forming stress fibers. Fibroblast cells cultured on artificial surfaces on tissue culture dishes develop also stress fibers while adapting to a two-dimensional growth environment (reviewed in Rönty, 2008).

2. The muscle cell

A contractile system involving actin and myosin is a basic feature of animal cells in general; however myofibrils of muscle cells display repeatable contraction and relaxation in a relatively short timescale. Cells specialized for contraction can be divided into skeletal muscle cells, cardiac muscle cells, smooth muscle cells, and myoepithelial cells. Skeletal and cardiac muscle cells appear striated, while smooth muscle and epithelial cells do not. Smooth muscles surround and control the involuntary movements of internal organs such as the large and small intestines, the blood vessels, and the uterus. Myoepithelial cells are found surrounding the secretory epithelium of glands or in the eye's iris (Reviewed in Alberts, 2002 and Mologni, 2009).

2.1. Striated muscle

Each skeletal muscle cell (fiber) develops by the fusion of several muscle precursor cells with a single nucleus called myoblasts. Myoblasts proliferate extensively, but once they have fused, they can no longer divide. Fusion generally follows the onset of myoblast differentiation, in which genes encoding muscle-specific proteins are switched on coordinately. Once formed, a skeletal muscle fiber generally survives for the entire lifetime of the animal. Skeletal muscle fibers secrete myostatin to control their own growth. Some myoblasts persist in a quiescent state as satellite cells in adult muscle and can be reactivated to replace damaged muscle cells after injury (Lee & McPherron, 1999, Seale et al., 2000).

Skeletal muscle is the most common type of muscle tissue in the body. It can be found in both slow and fast twitch forms. Fast twitch muscles can produce a burst of high energy for rapid and powerful movement, but they tire quickly. Their fibers are large in size and contain high glycogen storages and high glycolytic activity and narrow Z-disks. Slow twitch muscles produce less energy, but are designed for endurance and sustained work. They are richer in myoglobin, have high oxidative metabolism, and wide Z-disks (Luther et al., 2000).

The skeletal muscles are voluntary muscles, which allow for the movement of bones and joints, while cardiac muscle cells, cardiomyocytes, are involuntary and found only in the heart. Both skeletal and cardiac muscles are striated muscles composed of thousands of contractile units known as sarcomeres. Each sarcomere is composed of repeated ordered arrays of thin and thick filaments, which give the muscle a striated appearance when it is viewed in an electron microscope. However, the organization in the cardiac muscle is not as regular as that in the skeletal muscle. Also other differences between cardiac and skeletal muscle occur. The cardiac muscle is made of single cells (cardiomyocytes), each with mainly one centrally located nucleus. The cardiac muscle is composed of branched muscle fibers, which are interlocked with those of adjacent fibers by adherens junctions. These strong mechanical attachments enable the heart to contract forcefully without ripping the fibers apart. Together with desmosomes, the adherens junctions bind together the cell membranes of two adjacent cells at the intercalated discs. These discs facilitate rapid communication, allowing the heart to coordinate muscular contractions (Gutstein et al., 2003, Perriard et al., 2003). Because of this anchoring property, the sarcolemmal adhesions represent the focal sites for bidirectional transmission of intrinsically cell-generated and externally applied forces. For example, contracting adult rat cardiomyocytes plated on a laminin-coated silicone substrate produce pleat-like wrinkles on the substrate, which directly underlie the costameres (Danowski et al., 1992). Conversely, stretching rat cardiomyocytes end-to-end causes an immediate and homogenous increase in sarcomere length, indicating that externally applied strains are transmitted directly to the underlying contractile apparatus (Mansour et al., 2004). Cardiac muscle also differs from the other muscle types in that contraction can occur even without an initial nervous input. The cells that produce the stimulation for contraction without nervous input are called the pacemaker cells.

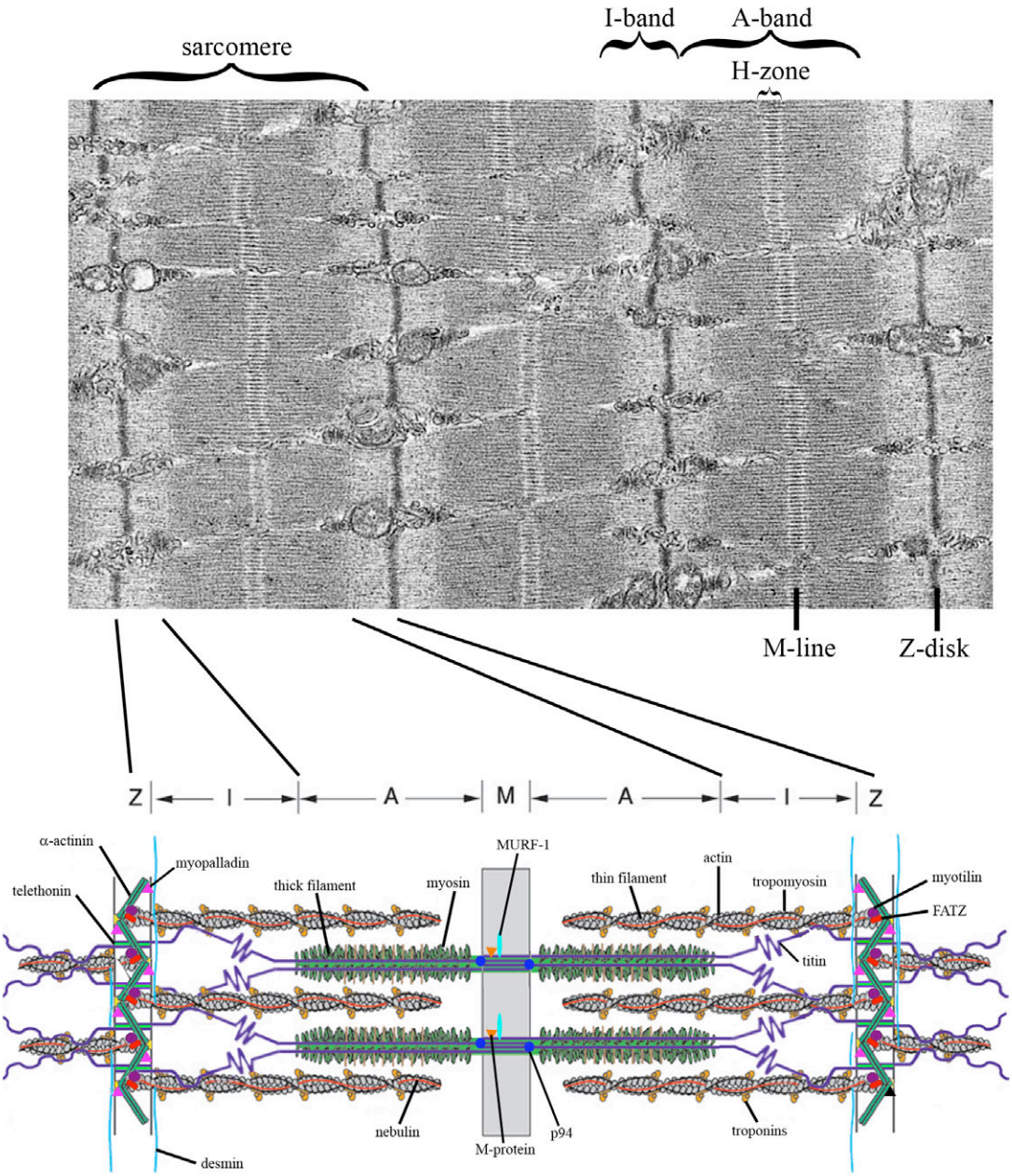

Figure 1. Electron micrograph (upper) and schematic drawing of a sarcomere (lower). *Thin filaments (chains of white round actin dots) are capped at the Z-disk by CapZ (yellow). Tropomyosin (black thread) and nebulin (red thread) filaments and troponin complexes (orange dots) are associated to the actin filaments. The Z-disk is composed of anti-parallel α-actinin molecules (dark green rods), myotilin (violet dot), FATZ (red rod), and myopalladin (pink triangle). The titin filament (violet thread) extends from Z-disk to M-line. At the Z-disk two titin filaments from opposed sarcomeres are cross-linked by telethonin (light green). The thick filaments are composed mainly of myosin (dark green). In the middle of the A-band, the M-line is composed of several proteins, among them M-protein (orange triangle), MURF-1 (light blue rod) and the calpain 3 enzyme (blue dot). (adapted from Moza, 2008, academic dissertation)*

2.2. Sarcomere structure and function

Apart from their role as force conduits, sarcolemmal adhesions initiate the assembly of sarcomeres. Sarcomerogenesis visualized in embryonic cardiomyocytes demonstrates that sarcomere precursors originate near the cell membrane at the sites of sarcolemmal adhesions (Du et al., 2008). Moreover, disruption of sarcolemmal adhesions results in loss of striated muscle organization, reduction of contraction, or cell death.

The sarcomere is the contractile unit of striated muscle cells containing repeated ordered arrays of actin containing thin and myosin containing thick filaments. Muscles move when these filaments slide past each other. The force is generated by the myosin heads, which undergo an actin-activated ATPase cycle during which they form transient cross- bridges between thin filaments in the regions of overlap (reviewed by Geeves & Holmes, 1999).

The sarcomere is divided into different bands or lines. A-bands span the length of thick filaments, while I-bands cover the area of thin filaments alone. The myosin part, which does not overlap with actin, is called the H-zone. The Z-disk (Z-line, Z-band) is the end of the sarcomere, where actin filaments from neighboring sarcomeres overlap and the M-line (M-region) is in the centre of the sarcomere, where thick filaments are cross-linked (Clark et al., 2002). The thick filaments are bipolar assemblies formed mainly from specific muscle isoforms of myosin II. Myosin binding proteins-C and -H contribute to the thick filament structure via interactions with myosin and titin in the A-band of the skeletal (-C and -H) and heart (-C) muscle sarcomere (Flashman et al., 2004). Myomesin, M-protein (or myomesin 2), and myomesin 3 are the main components of the M-band expressed in different muscle types. They bridge myosin filaments and anchor titin at the centre, creating a complex network of stabilizing interactions (Schoenauer et al., 2008).

The Z-disks largely consist of α-actinin homodimers organized in an anti-parallel fashion and providing a backbone for the insertions of actin filaments, as well as nebulin and titin. Titin forms a continuous filament system in the myofibrils, with single molecules spanning from the Z-disk to the M-band in both skeletal muscle and heart (Tskhovrebova et al., 2010). Different isoforms of the largest protein (3.0–3.7 MDa) vary in the size and structure of the elastic I-band part of the molecule. The size and structure of the thick filament part of titin is conserved, which is consistent with the conserved structure of thick filaments in vertebrates. Titin's N-terminus is coupled via telethonin (T-cap) to muscle LIM protein (MLP), which is believed to be central to Z-disk-based mechanosensing (Knöll et al., 2002). Because A-band titin provides regularly spaced binding sites for myosin and myosin binding protein C, it may function as a molecular ruler that controls assembly and length of the thick filament. Titin consists mainly of about 300 immunoglobulin (Ig) and fibronectin (Fn) domains that give to the entire protein a "beads-on-the-string" appearance (Labeit & Kolmerer, 1995). A unique region with spring-like properties, designated the PEVK segment confers elasticity to the entire molecule. Due to the PEVK region, titin behaves like an extensible spring (Linke et al., 2002). The M-line region of titin contains a serine/threonine kinase domain that has been shown to phosphorylate the Z-disk protein telethonin probably regulating myofibril assembly (Mayans et al., 1998). Furthermore, titin kinase may play a role in embryonic

sarcomere development, specifically, integration of titin in the A band and sarcomere structure maintenance. It has also been proposed that titin kinase is a mechanosensor that regulates muscle protein expression in a strain-dependent fashion. Titin kinase has also been proposed to assemble an nbr1-based signalosome that communicates with the nucleus and modulates, in a stretch-dependent manner, protein expression and turnover (Lange et al., 2005). Finally, recent studies suggest that titin kinase affects cardiac contractility owing to decreased sarcoplasmic reticulum calcium uptake (LeWinter et al., 2010). Titin binds several proteins that have diverse roles in sarcomeric structure, protein turnover, biomechanical sensing, and signaling. This suggests that titin has complex and important integrative functions, represented diversely in the different isoforms.

Titin and nebulin together specify Z-disk width, with titin constructing the central region of the Z-disk, including the number and positions of α-actinin cross-links and nebulin beeing a Z-disk terminator determining the ending of the Z-disk structure and its transition to the I-band. Nebulin is also a giant protein (500-900 kDa). A single nebulin molecule spans the thin filament with its C-terminus anchored at the Z-disk and its N-terminal region directed towards the thin filament pointed end (Labeit & Kolmerer, 1995). It consists of 185 repeated domains arranged into super repeats. This precise arrangement is thought to allow each central nebulin module (M9-M162) to interact with a single monomer of the actin filament (Labeit & Kolmerer, 1995), and each nebulin super-repeat to associate with a single tropomyosin (Tm)/troponin (Tn) complex. Nebulin's extreme N-terminal modules M1–M3 contain a high-affinity binding site for the thin filament pointed-end capping protein tropomodulin (McElhinny et al., 2003). Tropomodulin, in addition to binding nebulin's N-terminus, binds actin and tropomyosin with high affinity and prevents actin filaments from elongating or shortening at the pointed end (dos Remedios et al., 2003). Nebulin plays a critical role in regulating thin filament length, since in its absence in knock out mice the average thin filament length is shorter and force is greatly reduced (Bang et al., 2006, Witt et al., 2006).

In the transverse direction, linkage of myofibrils at the Z-disks allows for lateral force transmission and limits the degree to which adjacent myofibrils translocate relative to each other during active contraction or passive stretch, thereby preventing damage to inter-myofibrillar membrane systems, such as T-tubules and the sarcoplasmic reticulum. The intermediate filaments are thought be one of the major elements responsible for maintaining the highly ordered myofibrillar alignment of striated muscle and for the precise positioning of intracellular organelles within the myofiber. Desmin intermediate filaments link Z-disks of adjacent myofibrils with the plasma membrane (sarcolemma) and other organelles within the cell (mitochondria and nuclei). The attachment of the sarcomeres to the sarcolemma occurs at the costameres, sub-sarcolemmal cytoskeletal complexes aligned with the Z-disk and M-line (Clark et al., 2002).

The subunit proteins of desmin filaments are elongated coiled-coils with extensive intermolecular ionic and hydrophobic interactions between individual subunits, giving rise to filaments with high tensile strength as well as plasticity. Nebulin is required to laterally link myofibrils at the Z-disk by desmin filaments; in the absence of nebulin myofibrillar connectivity is significantly reduced leading to Z-disk displacement (Bang et al., 2002). In addition to linking adjacent myofibrils, nebulin's C-terminus regulates Z-disk width. The

mechanism by which nebulin terminates the Z-disk might involve interaction between nebulin and Z-disk-localized CapZ. CapZ is a barbed-end actin-capping protein that binds near the C-terminus of nebulin (Pappas et al., 2008). When these structural functions of nebulin are absent, muscle weakness ensues, as is the case in patients with nemaline myopathy with mutations in nebulin. In addition to the structural roles, nebulin may control contraction by controlling access of myosin heads to the actin filaments and participate in signal transduction (Ma et al., 2002), and be involved in physiological calcium handling of the sarcoplasmic reticulum-myofibrillar system (Ottenheijm et al., 2008).

Nebulette is a smaller, cardiac-specific nebulin homologue. Similar to nebulin, nebulette binds actin, myopalladin, and tropomyosin and is critical for thin filament assembly, their spatial organization and the contractile activity. In addition, nebulette interacts with the Z-disk proteins ZASP and filamin C (Holmes et al., 2008).

The filamins are a family of high molecular mass cytoskeletal proteins that organize filamentous actin into networks. The human filamins consist of 3 isoforms, filamins A, B, and C, which share approximately 70% sequence homology. The significance of filamins A and B in human biology was identified in genetic diseases affecting the brain, bone, and cardiovascular system (Krakow et al., 2004). Filamin C is predominantly expressed in skeletal muscle cells, where localizes at the myofibrillar Z-disk, by binding to myotilin, FATZ, and myopodin, an F-actin-binding protein that was initially reported to be significantly downregulated in Duchenne muscular dystrophy (van der Ven et al., 2000, Faulkner et al., 2000, Linnemann et al., 2010), and at the sarcolemma by interacting with γ- and δ-sarcoglycans (Thompson et al., 2000). Therefore, it provides a direct link between the sarcolemma and the myofibrils and is thought to have an important function in signaling between the two compartments. Filamin C plays an important role in early muscle development and stabilization of the myofibrillar Z-disk (van der Ven et al., 2000, Dalkilic et al., 2006).

2.3. Myotilin/palladin/myopalladin protein family

2.3.1. Myotilin

Myotilin, the main subject of this dissertation, is a 57 kDa protein consisting of two Ig domains flanked by a unique serine-rich N-terminus and a short C-terminal tail (Salmikangas et al., 1999). On the basis of similarities in sequences and structure, the protein domains that form the immunoglobulin super family have been divided into V, C1, C2, and I sets. These differ from one another with respect to edge strands on each beta sheet, in how far strands extend toward the “top” of the domain relative to the cysteines in the B and F strands, and with respect to certain framework residues (Harpaz & Chothia, 1994). The Ig domains of myotilin, locating at amino acids 252-341 and 351-441, were predicted by sequence comparison to fold into seven β-sheets and to fall into the category of C2-type Ig-folds. The high-resolution structure of the first Ig-domain of myotilin

determined with solution state NMR spectroscopy exhibits, however, the I-type of Ig-fold, being intermediate between the V and C type (Heikkinen et al., 2009). I-type fold is also seen in the structures of palladin Ig-domains 1 and 2, which are available in the PDB database (PDB accession codes 2DM2 and 2DM3). According to structural similarity search on DALI server the five closest structures to myotilin Ig1 are found in titin, aortic preferentially expressed protein-1, telokin, palladin, and myomesin. These all are clearly I-type Ig-domains (Heikkinen et al., 2009).

Similar Ig domains are found mainly in sarcomeric proteins such as titin, filamin C, myomesin, M-protein, MyBP-C, myopalladin and palladin (Vinkenmeyer et al., 1993, Labeit and Kolmerer, 1995, Vaughan et al., 1993, Bang et al., 2001, Rönty et al., 2004). Most of these proteins have significant links to human disease (reviewed by Otey, 2009) and certain inherited forms of heart disease are associated with mutations affecting the Ig domains of either MyBP-C or titin, which suggests that Ig domains have a key role in maintaining sarcomere integrity (Watkins et al., 1995, Gerull et al., 2002, Gerull et al., 2006). By sequence comparison, the Ig domains of myotilin are most homologous to Ig domains 2 and 3 of palladin 90-92 kDa isofoform (Parast & Otey, 2000, Mykkänen et al., 2001) and Ig domains 4 and 5 of myopalladin (Bang et al., 2001). Together, these three proteins form a subfamily of actin-associated proteins. Ig-domains are important for protein-protein interactions and the Ig-domain-containing region in myotilin interacts with N-terminus of the Z-line protein filamin C (van der Ven et al., 2000) and the C-terminus of myotilin makes homodimers in solution. Results obtained from cell transfection experiments suggest that myotilin's Ig domains also participate in F-actin organization (Salmikangas et al., 2003).

Myotilin (myofibrillar titin-like protein) was originally identified as a binding partner for α-actinin in a yeast two-hybrid screen. The proteins co-localize at the sarcomeric Z-disks (Salmikangas et al., 2003) by interaction between myotilin's amino terminal region (amino acids 79–125) and the C-terminal EF-hand repeats 3 and 4 of α-actinin (Hauser et al., 2000, A. Taivainen, M. Rönty, O. Carpén, unpublished data). Myotlin binds also directly to the Z-disk proteins FATZ-1, FATZ-2 (Gontier et al., 2005), and telethonin (Suila, H.2006 ASCB annual meeting B190).

Myotilin binds F-actin directly at a 1:1 ratio and cross-links actin filaments into large stable bundles in vitro. In cultured cells, expression of myotilin results in a unique phenotype with a network of filaments consisting of F-actin and myotilin (Salmikangas et al., 2003). Furthermore, forced expression of myotilin in early times of muscle cell development leads to strong actin bundle formation, which prevents normal assembly of sarcomeres (Salmikangas et al., 2003). These actin-regulating properties of myotilin are rather unique, and suggest that myotilin may play a role in sarcomere organization. In addition, mutations in the myotilin gene (*MYOT*) can cause different forms of muscle disease, characterised clinically by progressive muscle weakness and sarcomeric disarray.

The myotilin gene is located on chromosome 5q31 and the coding sequence is composed of 10 exons. Myotilin is mainly expressed in adult striated muscles and nerves (Salmikangas et al., 1999), but low levels of myotilin are also detected in other tissues (Godley et al., 1999). In mouse and human embryos, myotilin is expressed in lung, liver, skin, cartilage, and most of the nervous system (Mologni et al., 2001). In muscles,

myotilin is predominantly found within the Z-disks, although it has been observed in the sarcolemma as well, which could be explained by the interaction with filamins providing a link between the cell membrane and the sarcolemma (Salmikangas et al., 1999, van der Ven et al., 2000, Gontier et al., 2005). The expression of myotilin begins at late stages of muscle cell differentiation, after expression of titin and other sarcomeric proteins, suggesting that myotilin is involved in the final alignment of myofibrils rather than in initial assembly of the Z-disk. Myotilin is highly conserved and similarly regulated between human and mouse (Mologni et al., 2005). The coding sequences of the myotilin gene are 80% identical and amino acid sequences show 90% identity (Mologni et al., 2001). The human myotilin polypeptide consists of 498 amino acids and the mouse ortholog of 496 amino acids.

Myotilin's roles in normal mammalian development and physiology remain somewhat undefined. The widespread developmental expression of myotilin suggests a relevant role in mouse development. Mutations in the human myotilin gene have also been implicated in three different muscle disorders, suggesting that expression of wild-type myotilin would be required for normal muscle development in mammals. Surprisingly, conditional myotilin knockout mice are born at normal mendelian ratio and appears healthy throughout their lives. A thorough analysis has not revealed any abnormalities in sarcomeric structure in either embryonic or adult mice, and neither muscle strength nor muscle performance is affected in the mice (Moza et al., 2007). Since myotilin is closely related to palladin and myopalladin, it is possible that these family members compensate for the absence of myotilin in the knockout mice. Even double mutant myotilin-null/200kDa-palladin-hypomorph mice do only develop a mild myopathy at old age (Moza, 2008). It may be necessary to develop a muscle-specific triple myotilin/palladin/myopalladin knockout mouse to understand myotilin's role in muscle development.

Whereas myotilin knockout mice are virtually normal, mice with introduced myotilinopathy patient mutations develop progressive myofibrillar pathology, indicating that dysfunctional myotilin is more harmful to muscle cells than loss of the protein. A transgenic mouse model expressing human myotilin carrying a myotilinopathy associated mutation T57I reproduces many of the symptoms and pathology associated with the myotilinopathies—Z-disk streaming, myofibrillar aggregation and muscle weakness. Centrally located nuclei are also observed, indicating regeneration and replacement of damaged myofibres. Protein aggregates derived from degenerating myofibrils reminds of the pattern found in myotilinopathy patients. The aggregates contain α-actinin, filamin C, desmin, titin and myosin and also transgenic myotilin (Garvey et al., 2006). Compared to single-transgenic mutant mice, double-transgenic mice overexpressing myotilin showed more severe muscle degeneration, enhanced myofibrillar aggregation, and earlier onset of aggregation (Garvey et al., 2008). These data suggest that strategies aimed at lowering total myotilin levels in myotilinopathy patients may be an effective therapeutic approach.

In non-diseased muscle affected by eccentric exercise myotilin is present in increased amount in lesions related to Z-disk streaming and events leading to insertion of new sarcomeres in pre-existing myofibrils and can therefore be used as a marker for myofibrillar remodelling. Interestingly, myotilin is preferentially associated with F-actin

rather than with the core Z-disk protein α-actinin during these events, suggesting that myotilin has a key role in the dynamic molecular events mediating myofibrillar assembly (Carlsson et al., 2007).

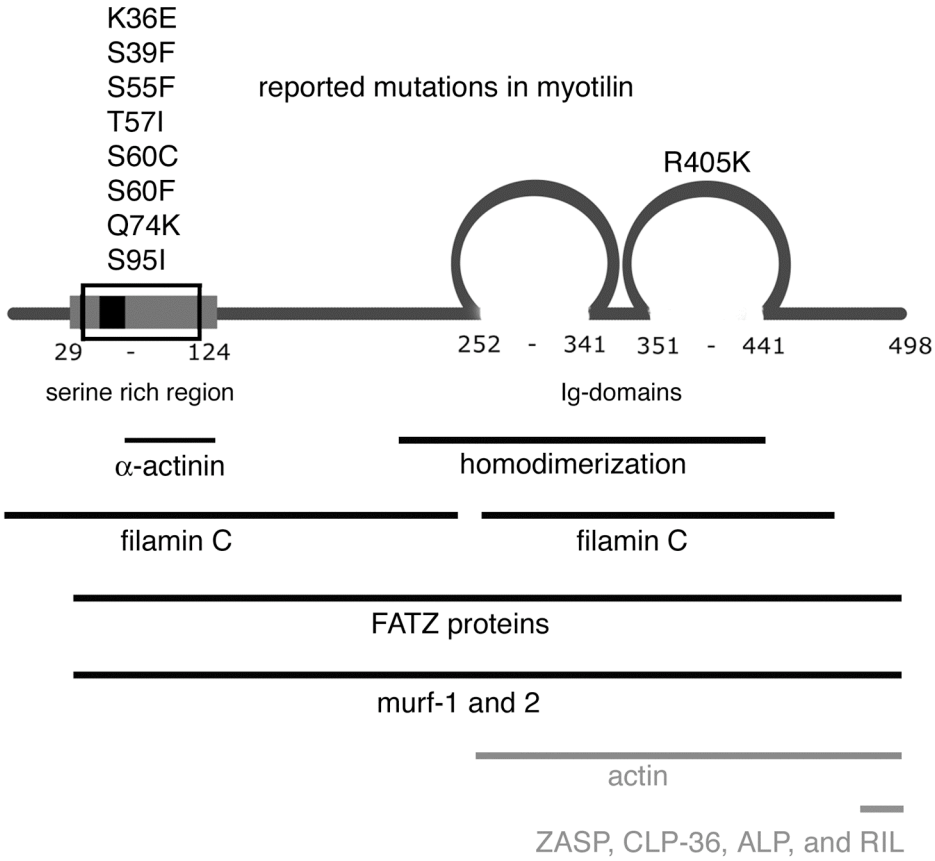

Figure 2. Domain organization of myotilin, reported myotilinopathy mutations, and interactions. *The molecule consists of a serine-rich region (grey box) containing a hydrophobic stretch (black box) followed by two Ig domains and a C-terminal tail. Reported myotilinopathy mutations are shown on top and regions of interaction with different interaction partners below. Interactions reported in this study are shown in grey.*

2.3.2. Palladin

Palladin can be described as an actin-binding molecular scaffold that forms complexes with a wide variety of cytoskeletal regulators. It was the second myotilin-palladin-myopalladin family member to be characterized, independently by three research groups (Parast & Otey, 2000, Liu et al., 2000, Mykkänen et al., 2001). In contrast to myotilin and myopalladin that are expressed predominantly in striated mucle, palladin is expressed in both muscle and nonmuscle cell types, and is especially abundant in embryos and neonates (Parast & Otey, 2000, Rachlin & Otey, 2006, Wang & Moser, 2008).

Palladin exists as multiple isoforms that arise from a single gene highly conserved between vertebrate species. Originally, three major isoforms (90 - 92, 140, and 200 kDa) were described, and these are transcribed from different promotors (Parast & Otey, 2000, Rachlin & Otey, 2006). Recently, up to fourteen potential isoforms have been annotated by various transcriptome databases (Otey et al., 2009). The high degree of isoform variability and tissue specific expression of isoforms suggest that different palladin variants may be specialized for different functions. The 90 - 92 kDa palladin doublet is the most widely expressed isoform, being essentially ubiquitous in developing mouse organs (Parast & Otey, 2000, Wang & Moser, 2008). Also the 140 kDa palladin is widely expressed, although it is not detected in several major organs such as liver, muscle and skin, while the 200 kDa isoform has been detected mostly in heart, skeletal muscle, testis, and bone (Rachlin & Otey, 2006, Wang & Moser, 2008). All palladin isoforms described contain from one to five Ig domains. The largest 200 kDa palladin isoform has two amino terminal and three carboxy terminal Ig domains, the 140 kDa variant has one N-terminal and three C-terminal Ig domains, and the smallest, most common 90-92 kDa isoform has three C-terminal Ig domains. The C-terminal Ig domains of palladin bind ezrin, a member of the ezrin-radixin-moesin family of scaffold proteins (Mykkänen et al., 2001) and bind and crosslink actin filaments (Dixon et al., 2008).

In addition to the Ig-domains, two proline-rich (PR) domains of palladin play important roles in its molecular binding interactions. The 90 - 92 kDa isoform has one PR domain, The 140 and 200 kDa isoforms two, that are located between the second and the third Ig domains of the 200 kDa isoform. Palladin's PR domains bind to Lasp-1, an actin-binding protein from the nebulin/nebulette family (Rachlin & Otey, 2006). Lasp-1 expression is required for normal cell migration, and misregulated Lasp-1 has been implicated in the motility of ovarian cancer and breast cancer cells (Lin et al., 2004, Grunewald et al., 2006, Grunewald et al., 2007). The PR domains of palladin interact also with the actin-regulating proteins VASP (and its relatives Mena, Ena, and EVL), profilin and Eps8 (Boukhelifa et al., 2004, Boukhelifa et al., 2006, Goicoechea et al., 2006). VASP and its relatives play important roles in actin cross-linking, regulating actin filament growth and cell motility (Boukhelifa et al., 2004). VASP forms complexes with profilin, suggesting that palladin and VASP may function together to recruit profilin to sites of actin polymerization (Boukhelifa et al., 2006). Eps8 plays a critical role in regulating the length of actin filaments, is involved in motility of invasive cancer cells, and is a substrate for the EGF receptor and many other tyrosine kinases (Goicoechea et al., 2006).

In addition, palladin's PR region binds to signaling intermediaries such as ArgBP-2 and SPIN-90 (Rönty et al., 2005, Rönty et al., 2007). Other interaction partners involved in signaling are Src, a key player in podosome formation (Rönty et al., 2007) and Akt-1 that mediates inhibition of breast cancer cell migration (Chin & Toker, 2010).

Like the other family members, palladin binds to α-actinin, a widely expressed actin-crosslinking protein, which docks in the region between PR2 and Ig-3 (Rönty et al., 2004). This binding sequence is highly conserved also in myotilin. Alpha-actinin is a member of the spectrin/dystrophin family, and it is ubiquitously expressed in vertebrate cells. Palladin co-localizes with α-actinin in focal adhesions, cell-cell junctions and stress fibres. In addition to binding F-actin, α-actinin also functions as a scaffolding molecule, and it

interacts with multiple transmembrane and signaling proteins (Otey & Carpen, 2004). Recently, palladin was shown to bind CLP36, mystique, and RIL, members of the alpha-actinin-associated LIM protein (ALP)/enigma protein family (Zheng et al., 2009, Hasegawa et al., 2010). Taken together, the diversity of palladin-binding partners suggests that palladin regulates the organization of the actin cytoskeleton via multiple molecular pathways.

In addition to being a molecular scaffold, palladin plays a role in cell motility, embryonic development, wound healing, and in invasive cancer (Goicoechea et al., 2008). Knockout of palladin in mice is embryonic lethal, demonstrating the importance of palladin in development (Luo et al., 2005). Mice with reduced muscle specific palladin 200 kDa isoform expression developed ultrastructural modifications in cardiomyocytes, but no skeletal muscle defects (Moza, 2008).

Mutations in the palladin gene (*PALLD*) have been connected to familial pancreatic cancer resulting from a single amino acid substitution at the α-actinin binding site (Pogue-Geile et al., 2006), although this finding has later been challenged (Zogopoulos et al., 2007). Palladin has also a connection to breast cancer, where palladin levels correlate with increased invasiveness (Wang et al., 2004).

2.3.3. Myopalladin

Myopalladin is the third member of the myotilin-palladin-myopalladin family and contains five Ig domains. Myopalladin's diverse molecular interactions suggest that it may be involved in both the structural aspects of sarcomere assembly and in regulation of sarcomeric gene expression. Myopalladin binds to nebulin and its relative nebulette. By its C-terminal region containing Ig domains, myopalladin binds to α-actinin and by its two N-terminal Ig-domain-region to the cardiac ankyrin repeat protein CARP (Bang et al., 2001). CARP localizes largely to the nucleus, where it regulates the expression of cardiac genes, and in the sarcomeric I-band. CARP is abundantly expressed in the developing heart and is strongly induced in cardiac hyperthrophy or stressed skeletal muscle (Aihara et al., 2000). Missense mutations in genes encoding for both CARP and myopalladin are associated with dilated cardiomyopathy (DCM) (Duboscq-Bidot et al., 2009, Duboscq-Bidot et al., 2008). In the myopalldin gene (*MYPN*), four independent missense mutations have been found to be responsible for DCM, suggesting that myopalladin plays a significant role in normal cardiac physiology. Three of the mutations are located in the C-terminal Ig domains of myopalladin and one of them, located in the fourth Ig domain, is associated with decreased localization to the Z-band area of left ventricular cardiac myofibrils (Duboscq-Bidot et al., 2008).

2.4. FATZ proteins

The FATZ (calsarcin, myozenin) proteins form another Z-disk family with structural and signaling functions. The three homologous members, FATZ-1 (calsarcin-2, myozenin-1),

FATZ-2 (calsarcin-1, myozenin-2), and FATZ-3 (calsarcin-3, myozenin-3) are localized in the Z-disk binding not only to myotilin but also to the filamins A, B, and C (Gontier et al., 2005), telethonin (T-cap), α-actinin, ZASP (cypher/oracle), and calcineurin (Faulkner et al., 2000; Frey et al., 2000; Frey & Olson, 2002; Takada et al., 2001). While FATZ-1 and FATZ-2 are expressed both in skeletal muscle and heart, FATZ-3 appears restricted to the skeletal muscle. The three proteins share high homology both at the N- and the C-terminal regions and in fact the binding sites for a variety of proteins occur in these areas. It has been suggested that the FATZ family may play a role in contributing to the formation and maintenance of the Z-disk (Frey & Olson, 2002) as well as in cell signaling since the members bind to calcineurin. Muscle cells are able to sense changes in their workload and adapt accordingly via complex signaling pathways, some involving calcium as its level in the muscle cells alters in response to the nerve pulses and muscle contraction. As a response to stress, muscle fibers hypertrophy, and become more efficient, shifting towards a slow fiber-type. Calcineurin is a sarcomeric calcium/calmodulin dependent phosphatase that could act as a sensor of change and is involved in the regulation of genes affecting muscle differentiation and fiber-type specification (Frey & Olson, 2002). FATZ-1 and FATZ-3 are highly expressed in skeletal muscle fast-twitch fibers, while FATZ-2 is highly expressed in cardiac muscle slow-twitch fibers. Mice lacking FATZ-2 show an increase in calcineurin activity and a concurrent increase in the percentage of slow-twitch fibers (Frey et al., 2004). Mutations in FATZ-2 are associated with hypertrophic cardiomyopathy (Osio et al., 2007).

2.5. PDZ-LIM domain proteins

Characterized by their Postsynaptic density 95, discs large and zonula occludens-1 (PDZ) and Lin-11, Isl1 and Mec-3 (LIM) domains, the PDZ-LIM family is comprised of evolutionarily conserved proteins found throughout the animal kingdom, from worms to humans. PDZ and LIM domains act as scaffolds, binding to filamentous actin-associated proteins, a range of cytoplasmic signaling molecules, and nuclear proteins during development and homeostasis (Krcmery et al., 2010).

PDZ domains are structurally conserved 80-100 amino acid modules being present singly or in multiple repeated copies in a diverse set of proteins. In most cases, they recognize C-terminal sequence motifs of target proteins and bind these peptides in a pocket between a β strand and an α helix (Harris & Lim, 2001). A given PDZ domain can interact with several targets. Similarly, a given PDZ binding motif of 3-7 amino acids can bind to several PDZ domains.

A classification of PDZ-binding motifs in the C-terminus has been used, in which the consensus sequence for type I is S/T-X-hydrophobic-COOH, and for class II is hydrophobic-X-hydrophobic-COOH (Songyang et al., 1997). More recently new PDZ-binding motifs, which do not belong to either of the two classes, have been discovered and it has become apparent that residues further N-terminal are important for specificity as well (Skelton et al., 2003, Beuming et al., 2005). Indeed, several different PDZ ligand

motifs seem to be needed since as many as 545 PDZ domains in 343 proteins are estimated (Zimmermann et al., 2006).

LIM domains can be found internally as well as near the N- or C-terminal regions of LIM domain proteins. The LIM domains are 50–60 amino acids in size and share two characteristic zinc finger folds, which are separated by two amino acids. The two zinc fingers that constitute a LIM domain contain eight conserved residues, mostly cysteines and histidines, which coordinately bond to two zinc atoms (Zheng et al., 2007).

There are seven PDZ-LIM proteins: PDLIM1/CLP36/ CLIM1/Elfin, PDLIM2/Mystique/SLIM, PDLIM3/ALP, PDLM4/RIL, PDLIM5/ENH, PDLIM6/LDB3/ZASP/Cypher, and PDLIM7/Enigma/LMP-1, the prototype of enigma gene family (reviewed by Zheng et al., 2009). They all localize to actin stress fibers or the muscle Z-disk. They have an N-terminal PDZ domain and one (ALP, RIL, CLP-36) or three (Enigma, ENH, ZASP/Cypher1) C-terminal LIM domains. PDZ-LIM proteins associate mainly with the actin cytoskeleton via their PDZ domain and with kinases via their LIM domain. The PDZ domains of many, if not all, of these proteins interact with the C-terminal peptide of α-actinin. In addition, ALP, ZASP/Cypher and CLP36 interact with the α-actinin rod domain via sequences located between the PDZ and LIM domains, mapping close to a conserved 26 amino acid motif, the ZM motif, found in these three proteins (Klaavuniemi & Ylänne, 2006). ALP, ENH and ZASP show high expression in muscle tissue, and CLP36 and RIL are expressed in various tissues, with high expression observed in epithelial cells (Vallenius et al., 2004). In muscle, PDZ-LIM proteins function as adaptors in translating mechanical stress signals from the Z-disk to the nucleus (Hoshijima 2006).

2.5.1. ZASP

Z band alternately spliced PDZ-containing protein (ZASP also named LIM domain-binding factor 3, Cypher, or Oracle) is a Z-disk-related cytoskeletal protein expressed in the striated muscles. Three groups found it independently in cardiomyocytes. The human and mouse sequences of ZASP were found by Faulkner's laboratory and named as Z band alternatively spliced PDZ-motif protein (Faulkner et al., 1999), Chen's laboratory identified splicing variants of mouse homologs of ZASP by in silico screening of LIM proteins enriched in the heart and named this gene as Cypher (Zhou et al., 1999), and Olson's group isolated mouse sequence of ZASP, named Oracle, during their process of differential screening of genes expressed specifically in the heart (Passier et al., 2000). There are several ZASP isoforms, all of which have an amino terminal PDZ domain required for binding α-actinin while the longer isoforms have LIM domains at the carboxy terminus involved in the binding PKCs (Zhou et al., 1999). This domain mediates interaction with ZASP in a phosphorylation-dependent manner and is involved in the targeting of ZASP. In mouse, six splice variants of ZASP/Cypher have been characterized, which fall into two classes, one specific to cardiac and the other predominant in skeletal muscle (Huang et al., 2003). These isoforms include short (Cypher2c, 2s) and long (Cypher1c, 1s, 3c, 3s) subtypes within both cardiac and skeletal muscle. Four human

splice variants of Cypher/ZASP have been identified, with one long and one short isoform specific to cardiac or predominant in skeletal muscle, respectively (Faulkner et al., 1999; Vatta et al., 2003).

ZASP knockout mice display a severe form of congenital myopathy and die postnatally (Zhou et al., 2001) and although ZASP is not required for Z-disk assembly it is required for the maintenance of the Z-disk during muscle function. Cardiac-specific ZASP knockout mice develop a severe form of DCM with disrupted cardiomyocyte ultrastructure and decreased cardiac function, which eventually lead to death before 23 weeks of age. A similar phenotype is observed in inducible cardiac-specific ZASP knockout mice in which ZASP is specifically ablated in adult myocardium. In the cardiac-specific knockout models, ERK and Stat3 signaling is increased (Zheng et al., 2009). In humans, ZASP is linked with dominant familial dilated cardiomyopathy (Vatta et al., 2003). An Asp626Asn mutation was demonstrated to increase the affinity of ZASP to PKC (Arimura et al., 2004) suggesting a disturbance of the adaptor function of ZASP for PKC may play a role in the pathogenesis of a subset of dilated cardiomyopathy. In addition to its association with DCM, mutations in ZASP result in myofibrillar myopathy (MFM) (Selcen & Engel, 2005; Vorgerd et al., 2005, Griggs et al., 2007).

2.5.2. ALP

The 36 kDa actinin-associated LIM protein (ALP, also known as PDZ and LIM domain protein 3 or PDLIM3) has an N-terminal PDZ domain and a single LIM domain at the C-terminus. Four ALP proteins have been identified in mammals, each having multiple splice variants and unique expression patterns (Zheng et al., 2010). ALP interacts directly with α-actinin and is co-localized with α-actinin at the Z-disks in cardiac or skeletal muscle (Xia et al., 1997), however, ALP localization at the Z-disk is independent of its association with α-actinin (Henderson et al., 2003). In fact, ALP is more readily detectable at the intercalated disks in adult mouse hearts in a distribution that does not overlap with α-actinin in cardiomyocytes (Pashmforoush et al., 2001). ALP is expressed in smooth, cardiac, and skeletal muscle cells and dramatically up regulated in differentiated smooth and skeletal muscle (Pomies et al., 1999 and Xia et al., 1997).

Mice that lack ALP develop right ventricular dysplasia and a mild right ventricular cardiomyopathy (Lorenzen-Schmidt et al., 2005, Lorenzen-Schmidt et al., 2000, Pashmforoush et al., 2001). ALP enhances the ability of α-actinin to crosslink actin filaments, indicating that ALP stabilizes actin filament anchorage at Z-lines and intercalated discs in cardiac muscle (Pashmforoush et al., 2001). Knockdown of ALP expression affects the expression of the muscle transcription factors Myogenin and MyoD, resulting in the inhibition of muscle differentiation (Pomies et al., 2007). These studies suggest that ALP plays a critical role in the integration of cytoskeletal architecture and transcriptional regulation during muscle development.

2.5.3. CLP36 and RIL

At the stress fibers, sarcomere-like structures in non-muscle cells with several shared components including myosin, tropomyosin, titin and α-actinin, 36 kDa C-terminal LIM domain protein (CLP36 also called CLIM1, Elfin, PDLIM1) and RIL (PDLIM4) bind α-actinin (Vallenius et al., 2004). CLP36 and RIL are mostly expressed in epithelial tissues and CLP-36 also in heart (Cuppen et al., 1998; Kiess et al., 1995; Vallenius et al., 2000, Vallenius et al., 2004). The comparison of *Clp36* and *Ril* expression patterns reveals that although they both are expressed in several epithelial tissues, the expression patterns do not overlap considerably, suggesting that they might have separate functions in cells (Vallenius et al., 2004). Both proteins have a PDZ domain at their N-terminal and a LIM domain at their C-terminal regions.

CLP36 associates with α-actinin 1 and α-actinin 4 at stress fibers in non-muscle cells (Vallenius et al., 2000) and with α-actinin 2 at the Z-lines in myocardium (Kotaka et al., 1999, 2000). CLP36 associates with Clik1, which is a serine/threonine protein kinase and is important for the localization of Clik1 to actin stress fibers (Vallenius & Mäkelä, 2002). CLP36 is also required for the organization of stress fibers and focal adhesions of BeWo (choriocarcinoma) cells (Tamura et al., 2007).

Ril was initially identified as a gene down-regulated in H-*Ras* transformed cells (Kiess et al., 1995), and RIL was shown to associate with the protein tyrosine phosphatase PTP-BL phosphatase via its LIM domain (Cuppen et al., 1998). Moreover, RIL interacts with the AMPA glutamate receptor in dendritic spines through the C-terminal LIM domain (Schulz et al., 2004). RIL homodimerizes through LIM-PDZ interactions (Cuppen et al., 1998), associates with α-actinin via its PDZ domain and enhances the ability of α-actinin to cross link F-actin. RIL over expression in cells leads to partially abnormal actin filaments showing thick irregular stress fibers not seen with CLP-36 and live cell imaging demonstrates altered stress fiber dynamics with rapid formation of new fibers and frequent collapse of thick irregular fibers in EGFP-RIL-expressing cells. These results implicate the RIL PDZ-LIM protein as a regulator of actin stress fiber turn over (Vallenius et al., 2004).

2.6. Sarcomere turnover and adaptation

During the continuous contraction of the muscle sarcomere, new proteins are exchanged into the structure via a carefully orchestrated process of synthesis and degradation. This continual remodeling allows adaptation to stress, including exercise, metabolic influences, or disuse and must occur without affecting the integrity of the contractile force necessary for the muscle to continue to function.

Striated muscle cells are almost crystalline in architecture and it is difficult to see how new elements might be added in a mature fiber under the constraint of continued force production by the muscle. Therefore, models of *de novo* sarcomere formation that follow the sequential assembly process of premyofibril formation initiated at the cell membrane may not be relevant to the adult cell remodeling in response to the stresses or strains

encountered in the body during adaptation to hemodynamic loads. Furthermore, sarcomere addition may proceed in either a longitudinal or transverse direction to regulate cell shape and function (reviewed in Russell et al., 2010).

Adult skeletal muscle is thought to lengthen mainly by the addition of new sarcomeres at the ends of the fibers near myotendon junctions (Dix et al., 1990). Fibers do not end at flat transverse discs, but at very irregular structures with some sarcomeres seen to creep ahead of others perhaps used to elongate the cell. The analogous terminal structure in the heart cell is the intercalated disc where force is transmitted longitudinally through very strong adherens junctions. Intercalated discs are even more irregular in cardiac hypertrophy and have a denser architecture in myopathic hearts (Perriard et al., 2003).

New sarcomeres can also be added in the middle of the fiber as shown in human skeletal muscle where a Y-shaped scaffold projects inwards from the membrane to splice in new, shorter sarcomeres at the Z-disk in a desmin-labeled zone (Yu & Russell, 2005). The role of a Y-scaffold for sarcomeric addition was also confirmed in cardiac myocytes during rapid lengthening in culture (Yu & Russell, 2005).

It is also possible that actin filaments and sarcomeres can be added internally well away from the membrane (Carlsson et al., 2007). The contractile material in striated muscle is thought by many to remodel using the Z-disk, which is a transversely oriented, lateral extension of the focal adhesion. Thus, Z-disks may act as a platform for actin filament polymerization internally in addition to the membrane location. Unfortunately this perpendicular structure is missing in conventional flat 2D cultures and its absence might explain slow progress in our understanding of myocyte width regulation. Hopefully this will be accelerated now with better 3D models in culture (Senyo et al., 2007) and from the zebrafish studies of Sanger and others (2009).

Factors that play a role in the regulation of protein quality control in the sarcomere include chaperones that mediate the assembly of sarcomere components and ubiquitin ligases that control their specific degradation. The co-chaperones UNC-45, Hsp90, and Hsp70 are required for the assembly of myosin and desmin assembly requires αB-crystallin (Barral & Epstein, 1999, Liu et al., 2008, Bar et al., 2004). There is clear evidence of sarcomere disorganization in animal models lacking muscle-specific chaperone proteins, illustrating the importance of these molecules in sarcomere structure and function. Furthermore, mutations in either desmin or αB-crystallin are the cause of numerous pathologies (Bar et al., 2004, Selcen 2010).

The dynamic interplay between sarcomere-specific chaperones and degradation of sarcomere proteins is necessary in order to maintain structure and function of the sarcomere. Muscle contains four proteolytic systems in amounts such that they could be involved in metabolic protein turnover: 1) the lysosomal system, 2) the caspase system, 3) the calpain system, and 4) the proteasome (reviewed in Goll et al., 2008). The catheptic proteases in lysosomes are not active at the neutral pH of the cell cytoplasm, so myofibrillar proteins would have to be degraded inside lysosomes if the lysosomal system were involved. Lysosomes could not engulf a myofibril without destroying it, so the lysosomal system is not involved to a significant extent in metabolic turnover of myofibrillar proteins. The caspases are not activated until initiation of apoptosis, and, therefore, it is unlikely that the caspases are involved to a significant extent in myofibrillar

protein turnover. It seems that both the calpains and the proteasome are responsible for myofibrillar protein turnover, but the mechanism is still unknown. The proteasome is responsible for over 80 to 90% of total intracellular protein turnover, but the proteasome degrades peptide chains only after they have been unfolded, so that they can enter the catalytic chamber of the proteasome. Thus, although the proteasome can degrade sarcoplasmic proteins, it cannot degrade myofibrillar proteins until they have been removed from the myofibril. It remains unclear how this removal is done. Calpains degrade those proteins that are involved in keeping the myofibrillar proteins assembled in myofibrils, and calpains can disassemble the outer layer of proteins from the myofibril and releasing them as myofilaments. Such myofilaments have been found in skeletal muscle (van der Westhuyzen et al., 1981, Goll et al., 2003). Individual myofibrillar proteins can also exchange with their counterparts in the cytoplasm (Peng & Fischman 1991, Swartz 1999), however, it is unclear whether this can be done to an extent that is consistent with the rate of myofibrillar protein turnover in living muscle.

The proteasome has a major role in intracellular protein degradation in all cells. The ubiquitin proteasome system involves specific ubiquitin ligases (designated E3) attaching poly-ubiquitin tails on targets for degradation by the 26S proteasome. For example, both MuRF1 and MuRF3 have shown to specifically ubiquitinate and degrade myosin in a proteasome-dependent manner in the heart and skeletal muscle (Fielitz et al., 2007). MuRF1 is reported to also interact with troponin T, myosin light chain-2, myotilin, and telethonin (Witt et al., 2005). The closely related MuRF2 protein also interacts with these aforementioned proteins *in vitro*, suggesting that a redundant system may exist for turning over these proteins. In addition, MuRF1, found mainly in the M-line of the sarcomere where it interacts with the giant protein titin, specifically recognizes and degrades troponin I in a proteasome-dependent manner. Muscle atrophy F-box/Atrogin-1, C-terminus of Hsp70-interacting protein, and Murine double minute 2 are additional muscle-specific ubiquitin ligases having a role in maintaining the sarcomere (Willis et al., 2009).

Calpains are required to mediate the dissociation of sarcomere proteins from the assembled myofibrillar structure before the ubiquitin-proteasome system (UPS) is able to degrade the sarcomere proteins (Neti et al., 2009). Calpains perform the initial proteolytic cleavage that allows E3 ubiquitin ligases to ubiquitinate the peptides and target them for degradation in the proteasome. Calpains do not degrade proteins to amino acids or even to small peptides and do not catalyze bulk degradation of the sarcoplasmic proteins, so they cannot be the only proteolytic system involved in myofibrillar protein turnover. No specific amino acid sequence is uniquely recognized by calpains. Amongst protein substrates tertiary structure elements rather than primary amino acid sequences are likely responsible for directing cleavage to a specific substrate.

Calpains are a group of calcium-dependent, non-lysosomal cysteine proteases expressed ubiquitously in all cells. There are more than a dozen calpain isoforms some with multiple splice variants. The two main isoforms, calpain 1 and 2 differ primarily in their calcium requirements (Goll 2003). Calpain 3 (or p94) is a more tissue specific protease expressed in muscle and brain (Beckmann & Spencer 2008, Konig et al., 2003). Disruption of the gene encoding calpain 3 has been shown to cause muscular dystrophy. Since the loss of calpain 3 results in muscle wasting, it seems unlikely that calpain 3 has a

general degradative role in skeletal muscle, but it acts rather as a signaling protease. Calpains are able to cleave many cytoskeletal proteins and can thus intervene in cytoskeleton regulation, particularly during processes such as adaptive response to exercise or regeneration after muscle wasting (Ojima et al., 2010).

A number of cytoskeletal proteins have been identified as potential calpain substrates *in vitro*, although not all have been confirmed as *in vivo* targets. These include myofibrillar/Z-disk proteins titin, α-fodrin, α-actinin, desmin, nebulin, filamin C, and myosin light chain 1, which supports the idea of calpain's role in sarcomeric remodeling (Barta et al., 2005, Beckmann & Spencer 2008, Murphy, 2010).

There is evidence that dysregulated protein turnover may play an important role in muscle or heart disease. When the calpain system is inhibited in the heart, as in mice over-expressing the endogenous calpain inhibitor calpastatin, morphological evidence of widespread protein aggregation has been identified along with increased autophagy (Galvez et al., 2007). The coordinated effort by calpain and ubiquitin ligases is also illustrated in models of skeletal muscle atrophy. Ubiquitin ligases, including MuRF1, have proven to be essential in the atropic process. When calpain inhibitors are introduced into the system, sarcomere degradation is inhibited, thereby inhibiting muscular atrophy, without reducing the ubiquitin ligase levels (Fareed et al., 2006).

3. Myopathies and muscular dystrophies

A myopathy is a muscular disease in which the muscle fibers are dysfunctional, resulting in muscular weakness. Muscle cramps, stiffness, and spasm can also be associated with myopathy.

A subgroup of myopathies, muscular dystrophies (MD), is inherited primary diseases of muscle, characterized pathologically by muscle fiber degeneration and clinically by muscle weakness. Classification of MD traditionally has been based on clinical, pathologic, and inheritance patterns (recessive, dominant, X-linked), but today gene mutation data have begun to dominate differential diagnostic methods (Rocha et al., 2010).

Nine diseases including Duchenne, Becker, limb-girdle, congenital, facioscapuohumeral, myotonic, oculopharyngeal, distal, and Emery-Dreifuss are always classified as muscular dystrophy but there are more than 100 diseases in total with similarities to muscular dystrophy (Kaplan et al., 2009, http://www.musclegenetable.org/). Most types of MD are multi-system disorders with manifestations in body systems including the heart, gastrointestinal and nervous systems, endocrine glands, skin, eyes, and brain. The condition may also lead to mood swings and learning difficulties.

The most common and severe form of the disease, Duchenne muscular dystrophy (DMD), is a severe recessive X-linked form of muscular dystrophy characterized by rapid progression of muscle degeneration, typically leading to walking disability by the age of 12 and eventually to death because of respiratory or heart failure. In general, only males are affected, though females can be carriers. The disorder is caused by a mutation in the gene coding for the dystrophin protein, an important structural component within muscle

tissue, providing structural stability to the dystroglycan complex (DGC) at the cell membrane. Mutations in the *dystrophin* gene can also cause a milder disease, Becker's muscular dystrophy (BMD), in which some patients can walk throughout their lives. The majority of DMD mutations cause the synthesis of a truncated, non-functional protein leading to loss of the whole DGC from the sarcolemma, resulting in instability of the muscle cell membrane, whereas mutations in the milder BMD leads to the production of a partially functional protein (Muntoni et al., 2003).

3.1. Limb-girdle muscular dystrophy

Limb-girdle muscular dystrophy (LGMD) is an autosomal class of muscular dystrophy that is similar but distinct from DMD and BDM. LGMD encompasses a large number of rare disorders with a significant genetic and clinical heterogeneity. The term limb-girdle describes the muscles most severely affected, the limb girdle muscles hips and shoulders.

Common symptoms of limb-girdle muscular dystrophy are muscle weakness, myoglobinuria, pain, myotonia, cardiomyopathy, elevated serum creatine kinase, and rippling muscles. The muscle weakness is generally symmetric, proximal, and slowly progressive (Laval & Bushby 2004).

The age of onset can be from early childhood to late adulthood and both genders are affected equally. When limb-girdle muscular dystrophy begins in childhood the progression appears to be faster and the disease more disabling than when the disorder begins in adolescence or adulthood (Laval & Bushby 2004).

The discovery of genetically distinct subtypes of LGMD has led to its current classification based on inheritance patterns, with the autosomal dominant form designated as LGMD1 and the most common forms of LGMD, the autosomal recessive LGMDs named LGMD2. The classical grouping of the LGMDs into LGMD1 and LGMD2 is being complemented by a classification based on the involved proteins and the underlying genetic defects. Autosomal dominant LGMDs comprise seven forms, often found in only a few families. Typically, mutations lead to the expression of dominant-negative forms of the involved proteins. LGMD1A-C are genes for known proteins, myotilin (LGMD1A), lamins A/C (LGMD1B), and caveolin-3 (LGMD1C) (http://www.musclegenetable.org/). Myotilin and myotilinopathy are discussed in separate sections (2.3.1 and 3.3). Lamins A/C are involved in various neuromuscular, cardiac, and lipodegenerative diseases (Mounkes et al., 2001) and mutations in lamins A/C are predicted to perturb the structure of the nuclear envelope (Brodsky et al., 2000). LGMD1B is the most common and clinically most variable form of LGMD1s (Rocha et al., 2010). Caveolin-3 is the muscle-specific member of the caveolin family of membrane proteins. Caveolins oligomerise in certain areas of the cell membrane, and constitute the coating of small membrane invaginations called caveolae, involved in membrane trafficking and cell signaling (Laval et al., 2004). Caveolin-3 is localized to the sarcolemma where it binds β-dystroglycan and dysferlin. Mutant caveolin-3 protein acts as a dominant negative inhibitor, oligomerizing with wild-type caveolin-3 and directing these complexes to proteasomal degradation leading to loss of caveolin-3 (Fulizio et al., 2005). At least four additional loci, LGMD1D-

G, have been described, but the causing genes have not been identified (Kaplan et al., 2009, http://www.musclegenetable.org/). In general, dominant forms tend to present after the second decade of life. Except for a few cases with rapid progression, the course usually is slowly progressive.

Compared with the dominantly inherited LGMD1 group, most autosomal recessive LGMDs have an earlier onset, rapid progression, and relatively high creatine kinase values. There are 15 currently known LGMD2s. LGMD2A, or calpain 3 deficiency, is the most common form of recessive LGMD (Rocha et al., 2010). Calpain 3 localizes to the skeletal muscle myofibrils by binding to titin (Beckmann et al., 2008, König et al., 2003). Titin has been proposed as a possible stabilizing agent that would prevent calpain 3 degradation in muscle tissues (Sorimachi et al., 1993). Calpain 3 differs from the ubiquitous calpains 1 and 2 by several specific regions that confer its unique functions and by a monomeric structure. Calpain 3 differs also from most of the remaining calpains by working at physiological, submicromolar calcium concentrations, by turning over rapidly, and by a proteolytic activity that is not inhibited by the ubiquitously expressed calpastatin (Beckmann et al., 2008).

There are no particular mutational calpain hot spots. The most common mechanism of LGMD2A is a deficiency in calpain 3 proteolytic activity, although there is a great heterogeneity in the LGMD2A associated calpain 3 mutations. The disease manifests in complete lack of calpain 3 expression, mislocalization of the protein, loss of enzymatic activity, or impairment of substrate cleavage or calpain 3 may simply perform other as yet unidentified non-proteolytic roles. Stretch-dependent calpain 3 distribution in sarcomeres plays a crucial role in the pathogenesis of LGMD2A, demonstrated with mice having endogenous calpain 3 replaced with a proteolytically inactive but structurally intact mutant protein (Ojima et al., 2010). There seem to be a correlation between type of mutation and phenotype (Kramerova et al., 2007, Beckmann et al., 2008).

LGMD2B is caused by mutations in the dysferlin gene (DYSF), coding for a protein involved in membrane repair (Bashir et al., 1998). LGMD2C-F are all the result of mutations in one of the four sarcoglycans (α, β, γ, δ) (Ozawa et al., 2005). Sarcoglycanopathies mimic dystrophinopathies (DMD and BMD). The sarcoglycan complex stabilizes the association of dystrophin with dystroglycan and the extracellular matrix. The integrity of this complex contributes to the stability of the plasma membrane cytoskeleton (Thompson et al., 2000).

LGMD2G is caused by mutations in the gene coding for telethonin, also called T-Cap. Telethonin is a titin capping protein at the myofibrillar Z-disk of the mature sarcomere, where it binds to FATZ-1, MLP and other proteins. During myofibrillogenesis, telethonin co-localizes with the M-band region of titin and is phosphorylated by the titin kinase domain (Frank et al., 2006). The disorder is relatively mild and has been reported in only five families to date.

LGMD2H is caused by mutations in the *TRIM32* gene, which encodes for a protein involved in the ubiquitin-proteasome degradation pathway. Tripartite motif-containing 32 (Trim32) is an E3-ubiquitin ligase with anti-apoptotic properties and its over expression is associated to cancer development. Trim32 ubiquitinates α-actin *in vitro*, suggesting it's involved in myofibrillar protein turnover (Kudryashova et al., 2005).

LGMD2I is a common form of LGMD in northern Europe. The disease is the result of mutations of fukutin-related protein (FKRP), a glycosyltransferase involved in the O-linked glycosylation of α-dystroglycan (Brockington et al., 2001). Mutations in FKRP lead to a marked reduction of α-dystroglycan at the sarcolemma (Brown et al., 2004). LGMD2I forms together with LGMD2K and LGMDM-O a group of disorders called dystroglycanopathies. LGMD2K is caused by mutations in the *POMT1* gene, LGMD2M by mutations in the fukutin-encoding gene. LGMD2N is associated with mutations in the POMT2 and LGMD2O with the POMGNT1 protein (Kaplan et al., 2009, http://www.musclegenetable.org/).

Titin is a sarcomeric protein connecting the Z-disk with the M-line and is associated with striated muscle development, structure, function and cell signaling. Tibial muscular dystrophy (TMD), a mild late-onset distal myopathy, results from heterozygous mutations of the titin (*TTN*) gene, whereas homozygosity for the same *TTN* mutation causes an autosomal recessive form of limb girdle muscular dystrophy (LGMD2J), with early onset and a more severe phenotype (Hackman et al., 2002).

LGMD2L has been linked to Anoctamin 5, also called TMEM16E or GDD1, is highly expressed in cardiac and skeletal muscle and in bone. The human Anoctamins compose a family of at least ten proteins of which some have been recognized as calcium-activated chloride channels, however, the function of Anoctamin 5 is unknown (Bolduc et al., 2010). The putative calcium-activated chloride channel function of Anoctamin 5 may be connected to deficient skeletal muscle membrane repair in muscular dystrophies.

Despite much progress over the last few years, the genetic cause of many cases of LGMD remains obscure and the classification of LGMDs is an ongoing process. Among the genetic causes of the autosomal dominant LGMDs, pure limb–girdle weakness appears to be a rather rare phenotype, whereas there is increasing awareness of presentations with distal myopathy or myofibrillar myopathy. The current treatment for LGMDs is supportive and aims at preventing complications, but gene therapy methods (exon skipping, stop codon read-through) are underway (Rocha et al., 2010). The major advances over the last two decades have improved diagnostic precision and focused symptomatic management and are increasingly leading to development of cutting-edge therapies.

3.2. Myofibrillar myopathy

Myofibrillar myopathies (MFMs) are morphologically distinct but genetically heterogeneous muscular dystrophies in which disintegration of Z-disks and subsequently myofibrils are followed by ectopic accumulation of multiple proteins as well as accumulation of degraded myofibrillar proteins forming large aggregates. MFM is characterized by slowly progressive weakness that can involve both proximal and distal muscles. Cardiomyopathy, neuropathy, and dominant inheritance are frequent associated features (Selcen & Carpen, 2008).

Table 1. Limb-girdle muscular dystrophies and Myofibrillar myopathies
(http://www.musclegenetable.org/, Mologni, 2009, Selcen, 2010)

NAME	HEREDITY	GENE PRODUCT /LOCUS	LOCALIZATION
LGMD1A	AD	myotilin	sarcomere
LGMD1B	AD	lamin A/C	nuclear membrane
LGMD1C	AD	caveolin-3	sarcolemma
LGMD1D	AD	6q23	
LGMD1E	AD	7q	
LGMD1F	AD	7q32	
LGMD1G	AD	4p21	
LGMD2A	AR	calpain 3	sarcoplasm/sarcomere
LGMD2B	AR	dysferlin	sarcolemma
LGMD2C	AR	γ-sarcoglycan	sarcolemma
LGMD2D	AR	α-sarcoglycan	sarcolemma
LGMD2E	AR	β-sarcolgycan	sarcolemma
LGMD2F	AR	δ-sarcoglycan	sarcolemma
LGMD2G	AR	telethonin	sarcomere
LGMD2H	AR	TRIM32	sarcoplasm/sarcomere
LGMD2I	AR	fukutin-related protein	sarcoplasmic reticulum
LGMD2J	AR	titin	sarcomere
LGMD2K	AR	POMT1	sarcoplasmic reticulum
LGMD2L	AR	Anoctamin 5	sarcolemma
LGMD2M	AR	fukutin	golgi
LGMD2N	AR	POMT2	sarcoplasmic reticulum
LGMD2O	AR	POMGnT1	sarcoplasmic reticulum
MFM	AD	desmin	sarcomere
MFM	AD	α-B crystallin	sarcoplasm/sarcomere
MFM	AD	myotilin	sarcomere
MFM	AD	ZASP (LDB3)	sarcomere
MFM	AD	filamin C	sarcomere
MFM	AD	Bag3	sarcomere

LGMD, Limb-girdle muscular dystrophy; MFM, Myofibrillar myopathy; AD, autosomal dominant; AR, autosomal recessive

Myofibrillar myopathy has also been referred to as desmin storage myopathy, desmin-related myopathy, or protein surplus myopathy. Because myofibrillar myopathy is genetically heterogeneous and the disease-causing protein or gene is known only in a minority of cases, because multiple other proteins besides desmin are also overexpressed in muscle, and because myotilin is not related to desmin, the noncommittal term "myofibrillar myopathy" is the preferred designation until the causative gene is determined. When the disease-associated gene/protein is identified, designations such as desminopathy, α-B crystallinopathy, myotilinopathy, zaspopathy, filaminopathy, or Bag3opathy are appropriate.

The genetic basis of myofibrillar myopathy has been elucidated in only about 20% of cases. Mutations have been identified in *DES*, the gene encoding desmin; *CRYAB*, encoding alpha crystallin B chain; *MYOT*, encoding myotilin; *LDB3 (ZASP)*, encoding LIM domain-binding protein 3; *FLNC*, encoding filamin C, and *BAG3*, encoding Bag3 (Selcen, 2010). These proteins are all associated with the sarcomeric Z-disk and have common mechanisms that lead to similar morphologic features of these protein aggregates.

3.3. Myotilinopathy

Myotilin is mutated in various forms of muscular dystrophy: LGMD1A, MFM, distal myopathy, and spheroid body myopathy (SBM). The first myotilin mutation was revealed at the same time as its genetic locus was identified and caused LGMD2 in two families through a T57I substitution in the N-terminal serine-rich region of the protein. This T57I mutation does not affect the expression level or localization of myotilin within the sarcomere, nor the interaction with α-actinin (Hauser et al., 2000). LGMD1A has later been described in other rare families with proximal muscular dystrophy combined with dysarthria and modestly raised serum CK (Hauser et al., 2002). Affected muscles show disorganization and streaming of the Z-line. As the diagnostic spectrum of myotilin mutations has been better defined, it is now clear that an "LGMD" presentation is probably a rare form of myotilinopathy (its more common presentation is with a distal myopathy). Presentation tends to be in middle to late adult life. This form of LGMD therefore overlaps with the MFMs, where the affected muscles may be distal, proximal or a mixture, with typically moderately raised serum CK (approximately five times the upper limit of normal) and characteristic histological features including vacuoles and accumulation of myofibrillar proteins such as desmin and myotilin. MFM patients may also have related cardiomyopathy and peripheral neuropathy, fitting well with adult expression of the *myotilin* gene. In addition to the T57I mutation, the mutations S55F, S60C, S60F, and S95I in the serine-rich region of myotilin were identified as the cause of LGMD1A or MFM (Selcen et al., 2004). These substitutions change a polar, potentially phosphoryable serine or threonine to a hydrophobic residue. Later, families with myotilin mutations (K36E, Q47K, and S60F) were found with both proximal and distal myopathy (Olive et al., 2005, Penisson-Besnier et al., 2006) or mostly distal myopathy (S55F, S60F) (Berciano et al., 2008, McNeill et al., 2009) confirming the broad clinical spectrum of

myotilinopathies. To date, only one myotilin mutation has been found outside the serine-rich region in one patient. The R405K mutation in the second Ig domain was shown to affects the homodimerization and α-actinin binding in yeast two-hybrid (Shalaby et al., 2009).

SBM has been described in only one family having the S39F mutation. The SBM patients have late-onset symptoms similar to LGMD1A, however, there is a unique pattern of spheroid bodies within the cytoplasm of type I fibers resembling the amorphous deposits observed in MFM patients (Foroud et al., 2005).

Although the exact mechanism of how the myotilinopathy mutations lead to a disease phenotype is unclear, the mutations seem to have a dominant negative effect on the protein function. The finding that forced expression of myotilin in early times of muscle cell development prevents normal assembly of the sarcomeres (Salmikangas et al., 2003) supports this hypothesis. Furthermore, while myotilin knockout mice lack an obvious phenotype and seem to have normal muscle function (Moza et al., 2007), a transgenic mouse strain expressing the mutated T57I human form of myotilin unites the disease phenotype (Garvey et al., 2006). The T57I mice develop myofibrillar pathology progressing with age, including Z-disk streaming, myofibrillar vacuolization and myofibrillar aggregation. Protein aggregates include transgenic myotilin, endogenous α-actinin, γ-filamin, desmin, titin, and myosin (Garvey et al., 2006).

Aims of the study

The aim of this study was to clarify the biological functions of myotilin and the molecular basis and the pathogenetic mechanisms that underlie myotilin-related diseases. Despite growing information on myotilin's properties, its role in muscle function and disease is still poorly understood. The specific aims were:

1) To study how myotilin regulates the actin cytoskeleton.

2) To determine myotilin's binding to Enigma proteins through a class III PDZ binding motif together with other myotilin and FATZ family proteins.

3) To study the turnover of myotilin and compare it with the turnover of mutated myotilin.

Materials and methods

Plasmids and Antibodies (I, II, III)

Full-length myotilin-encoding gene (residues 1-498) and its variants encoding amino acids 1-270 102-498, 185-498, 215-498, 347-498, 441-498, 217-250, 217-339, 217-439, 251-439, 347-439, 347-498 or palladin (residues 715-772) were PCR-amplified from human myotilin or palladin cDNA and subcloned into pGEX-4T1 GST-fusion vector (Pharmacia) for production of GST fusion proteins in bacterial cells and into pAHP vector (Salmikangas et al. 2003), pDEST27 (Invitrogen), pEGFP-C2 (Clontech) for mammalian expression or in vitro translation, and into EG202 and JG4-5 vectors (Gyuris et al., 1993) for yeast two-hybrid analyzes. The PDZ domain regions of human ZASP (1-255 bp), ALP (1-246 bp) and CLP-36 (1-255 bp) were PCR-amplified from their respective full length cDNAs; subcloned into pQE30 (QIAGEN) and a modified version of pGEX-6-P3 (GE Healthcare) for the expression of His and GST-tagged proteins, respectively. The full-length proteins of FATZ-1 and FATZ-2 were cloned as previously described (Faulkner et al., 2000). FATZ-3 (1-756 bp) was amplified by RT-PCR from human muscle mRNA (Clontech) and then cloned into pQE30 and the modified pGEX-6-P3. The site-directed mutations were generated by QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The authenticity of the constructs was verified by sequencing the relevant regions.

Myotilin was detected with a polyclonal rabbit antibody against myotilin residues 1-151 or 231-342 (Mologni et al., 2005), ZASP with a polyclonal rat antibody (Faulkner, unpublished), α-actinin-2 (Sigma) with a monoclonal mouse antibody (m Ab), ezrin with the m Ab 3C12 (Sigma Aldrich), and ubiquitin with a m Ab (Santa Cruz Biotechnology). Mouse anti-HA Ab was used to recognize the HA-tag (Roche or Nordic Biosite AB) and goat anti-GST Ab (GE Healthcare) for the GST-tag. Alexa 488-, 568-, and 594-conjugated goat anti-mouse and goat anti-rabbit Ab:s (Invitrogen; MolecularPprobes) were used as secondary antibodies and F-actin was visualized with Alexa Fluor 568 phalloidin (Invitrogen, Molecular probes) in immunofluorescence. Coverslips were mounted in DABCO (Sigma) and Mowiol (Calbiochem) and examined by immunofluorescence (ZEISS Axiophot equipped with AxioCam cooled CCD-camera) and confocal microscopy (Leica SP2 equipped with Ar and Kr lasers, Leica microsystems).

Cell transfections, treatments, and quantifications (I, II, III)

Rat cardiomyocytes were isolated using the neonatal cardiomyocyte isolation system (Worthington Biochemical Corporation) according to manufacturer's instructions, except that the tituration step was done twice. Cells were cultured in transfection medium (21% medium M199, 73% DBSS-K, 4% horse serum, 2 % L-glutamine; DBSS-K: 6.8 g/l NaCl, 0.14 mM NaH_2PO_4, 0.2 mM $CaCl_2$, 0.2 mM $MgSO_4$, 1 mM dextrose, 2.7 mM $NaHCO_3$)

for 2 h and transfected with Escort III (Sigma) according to the manufacturer's protocol. Then 6 h after transfection, the media was changed to maintenance medium (75 % DMEM, 19 % M199, 4 % horse serum, 2 % L-glutamine, 50 µg/ml gentamycin (Invitrogen), 0.1 mM Phenyl Ephrine (Sigma), 10 µM AraC (Sigma)). Mouse muscle cryosections were prepared as described (Moza et al., 2007). COS7 and CHO-cells (ATCC) and male rat kangaroo kidney epithelial cells (PtK2 Line: ATCC) were transfected with Fu-GENE6 reagent (Roche) and incubated 48 h before analyzis. C_2C_{12}-myofibroblasts (ATCC) were induced to differentiate into myotubes by shifting to culture medium containing 2% horse serum for 7-9 days.

Calpain activity was induced with the addition of 5 µM ionomycin (Calbiochem, Merck KGaA) and 10 mM CaCl2 for 1 h and calpain was inhibited with 10 µM Z-Leu-Leu-H (Z-LLal: PeptaNova GmbH) for 2 hrs. Protein synthesis was inhibited with 100 µg/ml cycloheximide (Sigma), proteolysis with 10 µM Z-Leu-Leu-Leu-al (MG 132: Sigma-Aldrich Chemie GmbH), proteasomal degradation with 7.5 µM lactacystine (Calbiochem), lysosomal degradation with 10 µM Bafylomycin A (Sigma-Aldrich), and cysteine proteases with 20 µM E64D (Sigma). Actin filaments were destabilized with 0.5 µM Latrunculin B (Sigma-Aldrich) for 7, 17, or 24 h. Cells were fixed, stained, and mounted as described (I, II).

The morphology of the phalloidin stained actin cytoskeleton was analyzed from 100 myotilin-transfected cells with different treatments and the experiment was repeated three times with similar results. The intensity of the Western blots were quantified by TyphoonImager 9400 (GE Healthcare) and analyzed by ImageQuantTL2003 software (GE Healthcare). Statistical analyzes were performed in Excel with Student´s t-test.

Transposon and generation of a pool of 15 bp insertion-containing mutant plasmids (I)

Plasmid pJGMyo was used as a target for pentapeptide insertion mutagenesis. pJG4-5(Δ*Not*I) was constructed by digestion of pJG4-5 with *Not*I and recirculation of the plasmid after filling-in with Klenow enzyme and dNTPs, eliminating a unique *Not*I site. The myotilin encoding region was also cloned into pJG4-5(Δ*Not*I) and this plasmid was used as a wt control for the created pentapeptide insertion mutants. The plasmid pSTH11, carrying the cat-Mu(*Not*I) mini-Mu transposon, has been described (Haapa et al., 1999). The linear cat-Mu(*Not*I) transposon was produced and purified as described (Haapa et al., 1999).

The *in vitro* transposition-based mutagenisation strategy exploits efficient Mu *in vitro* transposition reaction (Lamberg et al., 2002) in combination with custom-designed cat-Mu (*Not*I) mini-Mu transposon (Haapa et al., 1999). Standard *in vitro* transposition reaction (25 µl) contained 180 ng cat-Mu (*Not*I) transposon DNA as the donor, 850 ng plasmid pJGMyo as a target, 0.2 µg MuA, 25 mM Tris-HCl pH 8.0, 100 µg/ml BSA, 15% w/v glycerol, 0.05% w/v Triton X-100, 126 mM NaCl, and 10 mM $MgCl_2$. Six individual standard reactions were pooled, DNA extracted and resuspended in 20 µl water. Individual aliquots (1 µl per 25 µl cells) were used to electrotransform (Zou et al., 2003) competent

DH5α cells (Life Technologies). Transposon-containing plasmid clones were selected by ampicillin (Amp) and chloramphenicol (Cm) resistance. A total of ~1x105 colonies were pooled and grown in LB-Amp -Cm liquid medium (50 ml) at 37 °C for 2 h. Plasmid DNA from the pool was isolated, digested with *Eco*RI and *Xho*I and subjected to preparative electrophoresis on 0.6% agarose gel. The 3.2 kb DNA fragment pool, corresponding to transposon insertions into the myotilin-encoding DNA segment, was isolated and ligated into *Eco*RI-*Xho*I-digested plasmid pJG4-5 (Δ*Not*I). The ligation mixture was electrotransformed as above into MC1061 cells (Invitrogen), and plasmid DNA was prepared from ~3x104 colonies as described above. Most of the transposon sequence was then excised by cleavage with *Not*I followed by preparative electrophoresis and isolation of the plasmid backbone as above and recircularisation by ligation at low DNA concentration (~0.1 ng/µl). Ligated plasmids were electroporated into DH5α cells and selected on LB-Amp plates. The final insertion mutant plasmid library contained ~3x104 clones.

Insertion positions were determined roughly by initial restriction analysis with *Eco*RI-*Not*I and *Not*I-*Xho*. Interesting phenotype-generating mutation constructs were sequenced with vector-specific 5' and 3' primers and with a myotilin primer corresponding to amino the acids 214 - 220 in the protein. The sequenced plasmids were individually transformed into *S. cerevisiae* cells for morphological analysis.

Protein purification (I, II, III)

The pGEX expression plasmids containing cDNAs for α-actinin2, myotilin, pallladin, FATZ-1, -2 and -3 with and without the last 15 bp were transformed into *E. coli* BL21 (pLysS) cells. The pQE expression plasmids containing the cDNAs for the PDZ region of ZASP, ALP and CLP-36 were transformed into *E. coli* M15 cells. Protein expression was induced with 0.3-1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3-4 h at RT, and GST- and His-tagged proteins were purified respectively with glutathione-Sepharose beads (GE Healthcare) and Ni-NTA resin (QIAGEN) according to the manufacturer's protocol for native protein.

For the actin bundling assay, each myotilin fragment was released from the GST fusion by incubation for 1 h at RT with 10 U of thrombin (Sigma) in cleaving buffer (150 mM NaCl, 2.5 mM $CaCl_2$ in 50 mM Tris, pH 8). Thrombin was removed from the samples by Benzamidine Sepharose 6B beads (Amersham Pharmacia Biotech). The fragment containing α-actinin repeats R1-R4 (lacking the actin-binding domain) was expressed and purified as described (Young et al., 1998).

Actin-binding assay (I)

Actin filaments were assembled from purified rabbit skeletal muscle actin in G-buffer (5 mM Tris, pH 8, 0.2 mM CaCl, 0.5 mM DTT, 0.2 mM ATP) by addition of 0.1 volume of

10 x polymerizing mixture (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) and incubated at RT for 1 h. Purified myotilin (1.5 µM), α-actinin (1 µM) (Cytoskeleton, Inc.) and α-actinin R1-R4 (1 µM) was added to the actin filaments (3 µM). Reaction volumes were equalized to 50 µl with G-buffer. The mixture was incubated at 25°C for 30 min and subsequently centrifuged at 14,000 ***g*** for 3 min. Under these centrifugation conditions, actin filaments remain in the supernatant, whereas F-actin bundles sediment. The pellet and supernatant was separated and their volumes were equalized with Laemmli buffer. The samples were analysed for protein content by SDS-PAGE and Coomassie blue staining. The relative amount of actin, that was precipitated or remained in the supernatant, was evaluated by the NIH-image program from SDS-gels from at least three independent experiments.

In vitro binding assay (II)

For the *in vitro* binding assay pCMV-myc-FATZ-3 or pAHP-myotilin or palladin plasmids were used as templates in a T3 or a T7-coupled rabbit reticulocyte transcription-translation system (Promega). Approximately 4 µg of GST-fusion proteins on glutathione beads were incubated with 15 µl of *in vitro* translated, ^{35}S-labelled protein in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 130 mM KCl, 0.05 % Tween-20. After washes with the same buffer, bound material was eluted by boiling in Laemmli buffer, subjected to SDS-PAGE and detected by autoradiography.

Yeast two-hybrid analysis and morphological observations of yeast phenotype (I)

Yeast transformation and mating as well as detection of protein interactions by β-galactosidase activity were previously described (Grönholm et al., 1999; Rönty et al.; 2007). The growth rate of myotilin expressing yeast-cells was measured or baits and preys were mated and replica tested for β-galactosidase activity to indicate the interaction between actin and the different myotilin constructs. The level of myotilin expression was verified by immunoblotting. Alternatively, the C-terminal myotilin cDNA, encoding for amino acids 102-498, was introduced by PCR and conventional cloning to a yeast two-hybrid bait plasmid pGBKT7. The bait was used in screening of a human skeletal muscle library in pACT2 (Clontech). Positive clones were sequenced.

To visualize F-actin and the cell wall, fixed cells were pelleted and incubated with 0.66 µM rhodamine phalloidin (Molecular Probes) and 0.1 mg/ml calcofluor in PBS for 1 h. Cells were washed with PBS and suspended in DABCO (Sigma) mounting solution. The phenotype of different yeast clones was determined by DIC and immunofluoresence microscopy (Zeiss Axiophot equipped with AxioCam cooled CCD-camera, Carl Zeiss Esslingen).

Bioinformatics (II)

The program to extract proteins from any database with the last 5 amino acids having the motif E[ST][DE][DE]L was written by Prof. G. Valle, Genome Research Group, CRIBI, University of Padova. The last 8 amino acids were considered but only the terminal 5 amino acids were given the following weightings: position 0 L = 2, position 1 E or D = 1, position 2 E or D = 1, position 3 S or T = 1 and position 4 E = 1. A score of 6 was given when all the criteria are met. This program was used to check the UniProt Knowledgebase Release 11.3 (UniProtKB/Swiss-Prot Release 53.3 of 10-Jul-2007 and the UniProtKB/TrEMBL Release 36.3 of 10-Jul-2007).

Peptides and AlphaScreen (II)

The peptides used for this study were synthesized by the ICGEB peptide synthesis service using a Gilson AspecXL SPE robot. The linker made up of two gamma amino-butyric acid units was 12.3 Å in length. The following peptides were used: Biotin-GABA-GABA-EpSEEE, -ESEEE, -EpSEEL, -ESEEL, -EpTEEL, -ETEEL, -EpSEDL,-ESEDL, -EpSEDL, -ESDEL and –EpSDEL. For competition experiments the same peptides without biotin but with GABA were used.

Experiments were done using 384-well plates (OptiPlate-384 white opaque, Packard BioScience) in a final volume of 25 µl per well. Both the GST detection and the Histidine detection Kits for AlphaScreen were used according to the manufacturer's specifications (Perkin Elmer). The acceptor and donor beads were used at a concentration of 0.02 µg/µl (6.5 pM). First the protein(s) to be tested are added to the wells and immediately the acceptor beads are added. The following steps were done in the dark; the plate was incubated for 30 min at RT before adding the donor beads; then for a further 3 hours after which it was kept for 15 min at 28˚C to equilibrate the temperature. The signal was read at 28°C using a Fusion Alpha™Multilabel Reader (PerkinElmer™) at 300 ms excitation, 700 ms emission.

When testing a protein for binding it is necessary to titrate it against its partner to establish the concentration of both proteins resulting in a significant value for the ratio of the signal (S) to noise (N), normally S/N 8-50. In every experiment negative controls without one or both proteins were used to give the noise (background) level and biotinylated GST (0.5nM) or biotinylated His (1nM) were used to as internal controls to normalize the signal. The experiments were repeated at least 3 times. In the competition experiments the binding proteins were first added to the wells at a fixed concentration that would result in binding in the absence of a competitor and then the protein used as competitor was added at decreasing concentrations. The results were plotted as the ratio of the signal in the presence of the competitor divided by that of the signal in the absence of the competitor. These experiments were repeated at least three times, the mean and the standard deviation from the mean were plotted.

The Ratio is calculated as the mean of the normalized Signal divided by the mean of the normalized Noise ie $R = \frac{Sm}{Nm}$

The confidence of the ratio is calculated from the standard deviation of the Signal and the Noise.

$$\Delta R = R\sqrt{\left(\frac{\delta Sm}{Sm}\right)^2 + \left(\frac{\delta Nm}{Nm}\right)^2}$$

Sm = normalized signal mean; dSm= mean standard deviation of normalized signal
Nm = normalized noise mean; dNm= mean standard deviation of normalized noise

TranSignal PDZ Array Domains (II)

The PDZ array membranes (Panomics) were used according to the protocols in the manufacturer's handbook; the biotinylated peptides or His –tagged purified proteins were used as ligands at 0.3 µg/ml and 15 µg/ml, respectively. After chemiluminescence the membranes were exposed to Hyperfilm for ECL (GE Heathcare).

Phosphorylation experiments (II)

Four µg of GST fusion proteins were used for each reaction. For phosphorylation studies with rat skeletal muscle extract, soleus and gastrocnemius muscle specimens were prepared freshly from rats, cut into 1-2 mm piaces, frozen in liquid nitrogen for 2 h, and maintained in - 80°C until needed. The protocol used for the CaM Kinase II phosphorylation assays is that described by Upstate Biotechnology, the only difference being that samples were analyzed by SDS-PAGE instead of using a scintillation counter. The CaM Kinase II enzyme used was from Upstate Biotechnology (catalog no.14-217, active, CaM Kinase II purified from rat forebrain). The protocol for the PKA phosphorylation assays is that described by Alfthan et al., 2004.

In vitro proteolysis with calpain 1 (III)

75 mg rat skeletal muscle was pulverized in a chilled mortar with liquid nitrogen and homogenized by sonication in ice-cold reaction buffer in the absence of Ca^{2+}. The crude homogenates were subsequently incubated as described previously (Barta et al. 2005). Calpain 1 proteolysis was initiated by the addition of 1 or 5 U calpain 1 (Calbiochem) to the reaction mixtures. After incubation for 1, 5, or 30 min, aliquots were collected and

immediately boiled in Laemmli buffer. Mixtures incubated in the presence of protease inhibitors 10 μM Z-LLal, 75 μM E-64d, 500 μM calpeptin (Calbiochem), 1 mM PMSF (Merck), 3 μM aprotinin, 5 μg/ml pepstatin A, 2 μg/ml leupeptin (Sigma-Aldrich), or in the absence of Ca^{2+} served as controls. Alternatively, C_2C_{12} cells differentiated for 7-9 days were lysed in ice-cold reaction buffer and incubated in the presence of 2.5 U calpain 1 with or without Z-llal or Ca^{2+} for 30 min in 30°C. Incubations in the absence of calpain 1 were used to test the endogenous Ca^{2+}–dependent proteolytic activity.

GST-myotilin fragments were expressed in *E. coli* DH5α and purified as described previously (I). Glutathione-sepharose beads with 4 μg fusion protein were incubated with 0.4 U of recombinant, active calpain 1 (Human erythrocytes: Calbiochem) in 30 μl calpain buffer (20 mM Tris-HCl, 30 μM $CaCl_2$ pH 7.4) with or without 10 μM calpain inhibitor Z-LLal for 5 min at RT. A mixture without calpain served as control. Adding 25 μl of Laemmli reducing buffer stopped the reaction. Proteins were resolved in SDS-PAGE, blotted, and detected with myotilin antibodies or with silver staining. Equivalent loading of the GST fusion proteins was confirmed by immunoblotting with goat anti-GST antibody. Fragments including GST and amino acids 217-250 or 217-339 of myotilin were alternatively analyzed by mass spectrometry.

MALDI-TOF analyses (III)

The calpain digestion reaction was made directly on MALDI target plate. Then, a saturated matrix solution α-cyano-4-hydroxy cinnamic acid (CHCA) (Sigma) in 33% ACN/0.1% TFA was added. MALDI-TOF analyses were carried out with Autoflex III (Bruker Daltonics) equipped with a SmartBeam™ laser (355 nm), operated in positive and reflective modes. Typically, mass spectra were acquired by accumulating spectra of 2000 laser shots and up to 10 000 for MS/MS spectra. External calibration was performed for molecular assignments using a peptide calibration standard (Bruker Daltonics).

Peptide identifications were performed by searching the peptide monoisotopic masses for Peptide Mass Fingerprints or the amino acid sequence tag for peptide fragments in MS/MS against NCBInr database using Matrix Science's Mascot (http://www.matrixscience.com/, Matrix Science Ltd) / or against locally created databases in an intranet server. FlexAnalysis™ and Biotools™ softwares (Bruker Daltonics) were used to analyze MS data as search engine interface between raw data transfer and the databases in mascot server, respectively. The following parameters were set for the searches; 0.1 Da precursor tolerance and 0.5 Da MS/MS fragment tolerance for combined MS/MS searches, oxidized Met was set as variable modification, the enzyme was set to none.

Results and discussion

Myotilin binds both G- and F-actin *in vitro* (I)

Myotilin localizes to structures that contain contractile bundles of actin filaments, such as stress fibers in transfected fibroblast cells and sarcomeres in muscle cells. Previous studies had shown that myotilin binds F-actin directly at a 1:1 ratio and cross-links actin filaments into large, stable bundles *in vitro* seen in electron microscopy. In cultured cells, expression of myotilin results in a unique phenotype with a network of filaments consisting of F-actin and myotilin. Furthermore, forced expression of myotilin in early times of muscle cell development leads to strong actin bundle formation, which prevents normal assembly of sarcomeres (Salmikangas et al., 2003). These actin-regulating properties of myotilin are rather unique, and these characteristics provided excellent possibilities to study actin regulation.

This study characterized further the interaction between myotilin and actin. To provide molecular insight into the myotilin-actin interaction, a number of truncated and mutated myotilin variants were prepared and the function of these fragments was studied by several different assays. The effect of various myotilin domains on myofibrillogenesis has previously been reported (van der Ven et al., 2000, Salmikangas et al., 2003) and therefore these studies focused on cells not expressing endogenous myotilin. Myotilin bound both G- and F-actin *in vitro*, shown by a G-actin pull-down assay and by an F-actin co-sedimentation assay. Binding efficiency correlated with construct size, that is, longer fragments bound actin better. By recruiting G-actin to the Z-disks, myotilin could be an important player during initial steps of sarcomeric remodeling in myofibrillar alterations induced by eccentric exercise. The amounts of myotilin and F-actin are strongly increased in broadened sarcomeres after eccentric exercise, where new sarcomeres are inserted, while there is a temporary lack of α-actinin (as well as titin and nebulin) (Carlsson et al., 2006).

During yeast two-hybrid experiments, we noticed that yeast cells expressing myotilin showed retarded growth. Microscopic analysis demonstrated phenotypic alterations in the same cells. This led us to further analyze the effect of myotilin on yeast actin cytoskeleton and to develop an assay for rapid screening of myotilin mutants with defective function. This assay combined *in vitro* DNA transposition-based peptide insertion mutagenesis with phenotype analysis in yeast cells. We found that myotilin induced exceptionally thick actin bundles that spanned throughout the chain of unseparated myotilin-expressing yeast cells. We are not aware of any other protein with such a strong actin-bundling effect in yeast. The phenotype resembled that seen in COS7 cells and, together with yeast two hybrid results, indicates that the binding and bundling properties of myotilin are conserved between mammalian and yeast actin. The fact that myotilin-induced changes were also seen in the actin phenotype mutant yeast strains, in which important actin-binding proteins (tropomyosin, Sac 6 [fimbrin], Abp1, Cap2, coronin, Aip [actin-interacting protein], WASP) were knocked out, suggests that myotilin may be directly responsible for the actin-bundling effect.

Myotilin Ig-domains are important for interaction with actin (I)

We wanted to identify the specific region of myotilin responsible for actin binding. Analysis of the sequence of myotilin did not reveal any of the canonical actin-binding domains described for other actin-modifying proteins (dos Remedios et al., 2003). The shortest fragment of myotilin to bind actin was the second Ig-domain together with a short C-terminal sequence. However, both Ig-domains proved to be of functional importance both for actin binding and bundling. Deletion of either Ig domain did not abolish, but weakened actin binding both in the pull-down and transfection experiments. These results suggest that the Ig domains may bind actin separately, but the combined effect of both domains is needed for optimal myotilin–actin interaction. The result is in line with functional studies of other Ig domain-containing muscle proteins, in which tandem Ig domains are required for proper function. For instance, the interaction between titin and telethonin has been shown to depend on Ig domains Z1Z2 of titin. Neither an extension of the N- nor C-terminus of separate Ig domains induced telethonin binding, suggesting that the binding region was not located at the linker region between the two Ig domains (Zou et al., 2003). Myotilin 185–498 was the shortest construct to induce actin-bundling activity *in vitro* and *in vivo*. This result indicates that the mere actin-binding region is not sufficient for the bundling effect. Deletion of either Ig1 or Ig2 hindered actin bundle formation in cells but did not inhibit the dimerization of myotilin, suggesting that myotilin bundles actin through two actin-binding sites.

Through possible combinatorial interactions with additional actin and alpha-actinin binding proteins that localize to the Z-disk (filamin C, FATZ-1), myotilin is involved in actin stabilization and Z-disk function (Salmikangas et al., 2003, van der Ven et al., 2000, Gontier et al., 2005). Hence, myotilin mutations could interfere with the total actin-tethering capacity of the Z-disc. Neither the region in myotilin that binds α-actinin (amino acids 80-125) (Hauser et al., 2000) nor the disease-associated substitutions in myotilin, all of which reside between residues 55 and 95, were, however, required for actin bundling. This result suggests that the pathogenetic mechanism of myotilin mutations is independent of its actin-modulating effects.

Overall, our findings define the Ig-domain segment of myotilin as critical for the interaction with actin and indicate that the region of interaction is conserved between yeast and mammalian actins. Later, a kettin fragment containing only the four C-terminal Ig domains was shown to bind directly to F-actin (Ono et al., 2006), suggesting that binding of actin by Ig domains may be a highly conserved molecular mechanism shared by both vertebrate and invertebrate proteins. Recently also palladin, the closest homologue to myotilin, was shown to bind and bundle actin through two of its Ig-domains (Dixon et al., 2008). Traditionally, Ig domains are known to mediate protein–protein interactions, serve as dimerization sites, or act as modular “spacers” to place an interacting module in the correct position to perform its function. Our study, together with some other observations, indicates that one should also regard the Ig domain as an actin-binding domain. However, the precise mechanism is still unknown and needs further investigation. Also the structural data on the actin-binding motifs in myotilin and palladin are under investigation. The structure of myotilin’s first Ig domain was solved using solution state NMR spectroscopy

(Heikkinen et al., 2009), however, more structural information is needed to define the specific structural features that identify an actin-binding type of Ig domain.

ZASP is a new binding partner for myotilin (II)

Interactions between Z-disk proteins regulate muscle functions and disruption of these interactions results in muscle disorders. Dominantly inherited missense mutations in Z-disk components myotilin, myopalladin, ZASP (Cypher), and FATZ-2 can lead to disease of the skeletal muscle and/or the heart. To search for novel binding partners to myotilin, we screened a human striated muscle library with the yeast two-hybrid method using a C-terminal fragment of myotilin as bait and identified ZASP as a new binding partner of myotilin. The interaction was verified with *in vitro* and *in vivo* (rat cardiomyocytes) affinity assays and cell transfection experiments. We further mapped the regions mediating this interaction to the extreme C-terminus of myotilin and the N-terminal PDZ domain of ZASP. PDZ domains typically bind to motifs located at the extreme C terminus (Sheng et al., 2001) and containing a crucial C-terminal leucine. The C-terminal residue of myotilin is a leucine and thus part of a potential PDZ binding motif. When the leucine in myotilin was replaced by glutamic acid, binding was lost both to the full-length ZASP/Cypher and the ZASP/Cypher PDZ domain.

The myotilin and FATZ families share a conserved E[ST][DE][DE]L motif that mediates interaction with muscle-specific PDZ domains (II)

Together with our collaborators, we noted that the C-terminal 5 amino acids of myotilin share high similarity with palladin, myopalladin and the family of three FATZ (calsarcin/myozenin) proteins. This high similarity raised the question of whether all these proteins could interact via their C termini with the PDZ domain of ZASP or the PDZ domains of other proteins. We showed with bioinformatics that the C-terminal E[ST][DE][DE]L motif is present almost exclusively in the myotilin and FATZ protein families and is evolutionary conserved. Based on previous classification of PDZ-binding motifs (Hung & Sheng, 2002, Songyang et al., 1997), the C-terminal ligand motif of myotilin and FATZ family proteins characterized in this study can be considered as a novel type of class III PDZ binding motif.

We used affinity precipitation assays, colocalization studies, the quantitative AlphaScreen technique, and a PDZ domain array to show that proteins from the myotilin and FATZ families interact via this novel type of PDZ binding motif with the PDZ domains of ZASP and other Enigma family members: ALP, CLP-36, and RIL. The PDZ domain array demonstrated that the interaction of the FATZ and myotilin families with the Enigma family members is highly specific, since the only two Enigma family members, of the 28 PDZ domain proteins on the PDZ array, bound to the peptide ligands.

Consistent with previous results (Kim & Sheng 2004) we showed that the interactions between the PDZ domains and their ligands are modulated by phosphorylation. Except for the finding that Src phosphorylates palladin (Rönty et al., 2007), no information on the interplay between kinases and the myotilin or FATZ families was available. We showed that muscle lysate contains kinase activity that can phosphorylate myotilin and tested then whether kinases associated with muscle pathophysiology can phosphorylate the C-termini binding to Enigma family PDZ domains. Calmodulin-dependent kinase II phosphorylated the C-terminus of FATZ-3 and myotilin, whereas PKA phosphorylated that of FATZ-1 and FATZ-2.

In muscle, PDZ proteins function as adaptors in translating mechanical stress signals from the Z-disk to the nucleus (Hoshijima, 2006). Some members of the Enigma family of PDZ proteins, ZASP for example, are known to bind protein kinases via their C-terminal LIM domains. Therefore, it is possible that ZASP and some other Enigma family proteins link the proteins of the myotilin and FATZ families to signaling events such as PKC phosphorylation. In this study we demonstrated that several of the known disease-associated Z-disk proteins are part of the same structural complex, whose composition can be regulated by signaling molecules associated with pathophysiological stimuli.

This was the first report of a binding motif common to both the myotilin and the FATZ families that is specific for interactions with Enigma family members. Our results were confirmed by the studies from another group demonstrating the specific binding of ZASP PDZ domain to the C-terminal region of both FATZ-2 and myotilin within the Z-line (Zheng et al., 2009). Also the interaction between palladin and CLP-36 was later confirmed. The interaction was dependent on the PDZ domain of CLP-36 and the C-terminus of palladin, and silencing of palladin inhibited the localization of CLP-36 to stress fibers (Maeda et al., 2009). Another group showed by yeast two-hybrid that the PDZ domain of CLP-36 and the last three amino acids (EDL) were needed for the protein interaction. Also mystique and RIL, two other members of the ALP/enigma protein family, bound to the C-terminus of palladin (Hasegawa et al., 2010).

Myotilin is a substrate for calpain (III)

The morphological findings typical of myofibrillar myopathy (MFM) include Z-disk alterations and aggregation of dense filamentous material (Clemen et al., 2009). The causes and mechanisms of protein aggregation in myotilinopathies and other MFM patients remain unknown; however, impaired protein degradation may explain in part the abnormal protein accumulation. In myotilinopathy patients, myotilin containing filament aggregates are immunostained for ubiquitin and the biologically dysfunctional mutant form UBB^{+1}. Also the polyubiquitin-binding protein p62, a multimeric signal protein known to be involved in aggregate formation, is found in myotilin positive aggregates (Olivé et al., 2008).

To gain more information about the degradation of myofibrillar proteins, we studied the mechanisms that control myotilin turnover. Calpains are required to mediate the dissociation of sarcomere proteins from the assembled myofibrillar structure before the

ubiquitin-proteasome system is able to degrade them. Calpains perform the initial proteolytic cleavage that allows E3 ubiquitin ligases, MuRF1 for example, to ubiquitinate the peptides and target them for degradation in the proteasome. In this study, we reported that myotilin is a calpain substrate *in vitro*, in cells, and in muscle tissue. The interplay between myotilin and calpain is in line with their colocalization in the Z-band and under the plasma membrane in mouse skeletal muscle fibers (Raynaud et al., 2006).

Our results indicated that the rat muscle myofibril has the potential to modulate its proteins, myotilin for example, via its own calpains. In mature sarcomeres, myotilin co-localizes with α-actinin and Z-disk titin, showing the striated pattern typical of sarcomeric proteins. In skeletal muscle, calpain cleavage of myotilin could be required for reorganization of muscular fibres after eccentric exercise. Myotilin is present in increased amounts in lesions related to Z-disk streaming and events leading to insertion of new sarcomeres in pre-existing myofibrils induced by eccentric exercise (Carlsson et al. 2007). Myotilin is more associated to F-actin than to the core Z-disk protein α-actinin during these events and might dissociate from α-actinin by calpain cleavage. Goll et al. (1991) have suggested that calpain 1 may release α-actinin from the Z-line intact via the modulation of other interacting proteins. α-actinin has also been shown to be least susceptible to calpain 1 proteolysis of several myofibrillar proteins (Barta et al., 2005). Myotilin appears already during initial steps of the remodelling process, before α-actinin, titin and nebulin, to the new sarcomeres. The susceptibility to calpain 1 cleavage leading to further degradation and release of new building blocks, could explain the rapid turnover of myotilin when the level of calcium in muscle cells is high during muscle contraction. For example, in cultures of quail myotubes, myotilin has a fast recovery rate compared to six other Z-disk proteins by FRAP (Wang et al., 2005).

We identified two calpain cleavage sites in myotilin, one at the amino terminal side of Q226 and the other at the amino terminal side of I253, by mass spectrometry. There are no consensus sequences for calpain cleavage, but they usually cleave destructured regions and both calpain cleavage sites in myotilin resided at a destructured region before the first Ig-domain.

Degradation of myotilin by the proteasomal pathway (III)

We showed with proteasome inhibitors that myotilin is further degraded by the proteasome in transfected COS7 cells and in C_2C_{12} myotubes expressing myotilin. Treatment of myotilin expressing cells with proteasome inhibitors induced morphological changes where myotilin accumulated in aggregates or dots, which also contained F-actin. The dots concentrated around the membranes and the normal actin cytoskeleton was partially intact. This indicates that when the ubiquitin proteasome system is disturbed in cells, the turnover of myotilin is dysfunctional leading to protein accumulations containing myotilin and actin filaments. The turnover of myotilin in the myotubes with organized sarcomeres is slower than in the fibroblast cells. When the filamentous actin structures in the myotubes were disrupted with latrunculin B, the degradation of myotilin became faster. This is consistent with the idea that the myofibrillar proteins must be dissociated

from the myofibril before they can be degraded downstream to amino acids by the proteasome and cellular peptidases (Solomon & Goldberg, 1996).

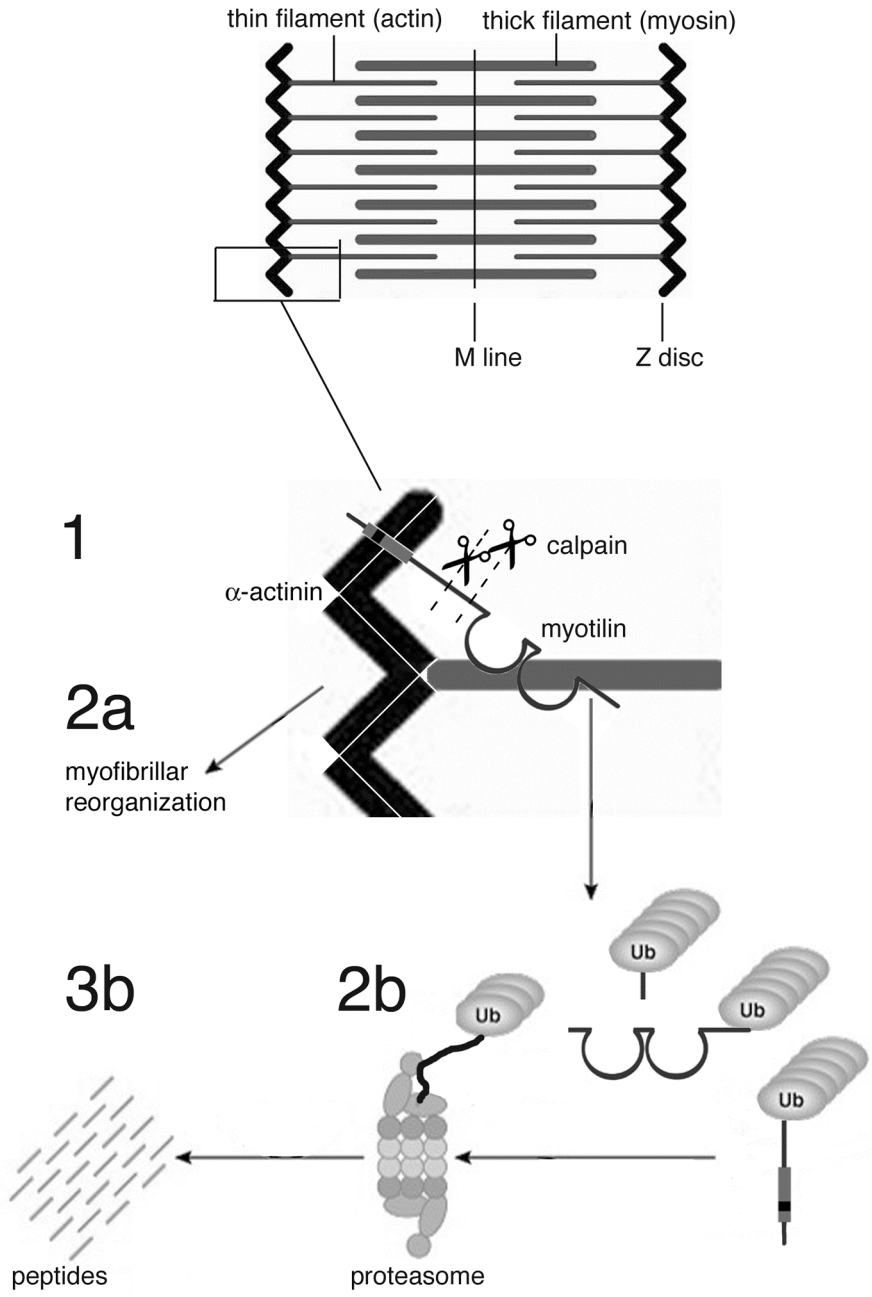

Figure 3. Schematic picture of events after calpain cleavage of myotilin in muscle cells. *Calpain cleavage of myotilin at sites amino-terminal to the first Ig-domain (1) leads to myofibrillar reorganization (2a). Alternatively, myotilin turnover is initiated by calpain cleavage. The cleavage is not affected by disease-causing mutations. Cleaved myotilin undergoes degradation via the ubiquitin-proteasome system (2b) (and additional mechanisms). Mutated myotilin is more resistant to degradation. This leads to accumulation of myotilin and induction of actin-containing protein aggregates (modified from III).*

Mutant myotilin is more resistant to degradation than wild type protein (III)

We demonstrated that proteins with myotilinopathy mutations degrade more slowly than wt myotilin. Previously, mutations in myotilin have been tested for their role in myotilin dimerization, interaction with α-actinin and actin, actin bundling, and myotilin phosphorylation, but the studies have not revealed differences with wild type myotilin. The results shown here, for the first time indicate a functional difference, as the patient mutations showed to be more resistant to degradation than the wt protein. We show that if the degradation of myotilin is disturbed, it accumulates in cells in a manner resembling that seen in myotilinopathy patients. This is supported by the fact that the amount of myotilin in the patient's muscle samples is increased (Barrachina et al., 2007, Shalaby et al., 2009). Based on the results, we propose a model on the pathogenic mechanism, by which myotilin mutations induce muscular dystrophy (Figure 3). In this model, mutated myotilin is more resistant to proteolytic breakdown, which leads to accumulation of myotilin and induction of actin-containing protein aggregates. Protein aggregation is not only a secondary defect, since we showed that cells transfected with GFP myotilin (in opposite to GFP transfected cells) induced protein aggregates after proteasome inhibition. The aggregates may become toxic when sequestering essential cellular proteins and eventually cause myopathy by disrupting the myofibrils. In addition, mutant myotilin may cause myopathy by still unknown mechanisms leading to disorganization of the Z-disk.

Conclusions

In conclusion, this study gave new information about myotilin's interaction with actin and identified new interaction partners, ZASP, CLP-36, ALP, and RIL. All, except RIL, are components of the muscle Z-disk. Our aim was to get more information about myotilin, which in our laboratory had been identified as an α-actinin binding protein in human striated muscle (Salmikangas et al., 1999). We developed various cell culture models, functional assays, recombinant proteins and synthetic peptides to aid in understanding how myotilin functions in muscle. In the beginning of our studies, a missense mutation T57I in myotilin was known to cause one form of skeletal muscle disease, LGMD1A (Hauser et al., 2000). Later, more mutations were found that substituted a serine or threonine for another amino acid in the protein causing different muscle diseases, now collectively termed myotilinopathy. The α-actinin-binding site in myotilin resides in the N-terminal region where the patient mutations were found. Therefore, we studied how deletion or mutation of this part in myotilin affects the actin-organizing properties of myotilin. We showed that neither α-actinin-binding nor actin bundling was affected by the mutations in myotilin. Another hypothesis was that myotilin would be phosphorylated at the serine-rich region and that the mutations would affect this phosphorylation. We, however, did not find any phosphorylation of myotilin in the serine-rich area.

Except for the finding that Src phosphorylates palladin (Rönty et al., 2007), no information on the interplay between kinases and the myotilin or FATZ muscle protein families was available. In this study we showed that the interaction between myotilin and the Z-disk ptoteins ZASP and CLP-36 is regulated by phosphorylation. Many Z-disk proteins have been found since this study began. At that time the sarcomere was thought to be a static structural unit and myotilin was defined as a core structural protein. Now it is known that the nature of the Z-disk allows it to serve both as a structural unit and as a coordinator of intracellular signaling. The Z-disk can respond to external stimuli by dissolution and reorganization, which requires dynamic and coordinated dissociation and association of molecular interactions. Phosphorylation of myotilin as well as palladin and the FATZ family proteins, shown in this study, is one way of regulating the protein-protein interactions in the sarcomere. In addition, it is possible that ZASP and some other PDZ-domain-containing proteins link the proteins of the myotilin and FATZ families to signaling events such as PKC phosphorylation. Based on the work presented here, we propose that the FATZ and myotilin families bind to members of the Enigma family of PDZ proteins (ZASP, CLP-36, ALP, and RIL) via the E[ST][DE][DE]L C-terminal ligand motif that can be considered a novel class III PDZ domain binding motif. Hence, we demonstrate that several of the known disease-associated Z-disk proteins (myotilin, myopalladin, FATZ-2, and ZASP) are part of the same structural complex, whose composition can be regulated by signaling molecules associated with pathophysiological stimuli. Since mutations in these proteins can lead to myopathies, knowledge of their protein interactions is highly relevant to understanding muscle and cardiac disorders.

Myotilin, palladin and myopalladin form a subfamily of Ig-domain-containing actin-associated proteins, but their actin-binding motifs were not known. Analysis of the sequence of myotilin did not reveal any of the canonical actin-binding domains described

for other actin-modifying proteins (dos Remedios et al., 2003). Here we identify the Ig-domains of myotilin as actin-binding motifs also important for actin bundling. Later, also palladin, the closest homologue to myotilin, was shown to bind and bundle actin through two of its Ig-domains (Dixon et al., 2008). However, the precise mechanism is still unknown and needs further investigation. Also the structural data on the actin-binding motifs in myotilin and palladin are needed to identify the specific structural features that define an actin-binding type of Ig domain.

The likely mode of pathogenesis in myotilinopathies is via a dominant negative effect i.e., the production of "poison" proteins that interfere with the normal function of the native myotilin. While myotilin knockout mice lack an obvious phenotype and seem to have normal muscle function (Moza et al., 2007), a transgenic mouse strain expressing a patient mutation form of myotilin unites the disease phenotype (Garvey et al., 2006). The transgenic mice develop myofibrillar pathology progressing with age, including Z-disk streaming, myofibrillar vacuolization and myofibrillar aggregation. Protein aggregates include transgenic myotilin, endogenous α-actinin, γ-filamin, desmin, titin, and myosin. Impaired protein degradation may explain in part the abnormal protein accumulation in diseased cells. Therefore, we studied the mechanisms that control myotilin turnover. We obtained evidence that myotilin is a calpain substrate and identified two calpain cleavage sites in myotilin. In skeletal muscle, calpain cleavage of myotilin could be required for reorganization of muscular fibres or for the degradation of myotilin. Based on our findings, calpains could mediate the dissociation of myotilin from the assembled myofibrillar structure before the ubiquitin-proteasome system is able to degrade the protein. We showed that myotilin was further degraded into small peptides by the proteasomal machinery and when this machinery was inhibited, myotilin aggregated together with actin in the cells. It would be interesting to test whether these kinds of accumulations contain additional proteins aggregated in myotilinopathy and other MFM patients. We demonstrated that proteins with myotilinopathy mutations degrade more slowly than wt myotilin. The results shown here, for the first time indicate a functional difference between wild type and mutated protein, since the patient mutations were more resistant to degradation than the wild type protein. We showed that if the degradation of myotilin is disturbed, it accumulates in cells in a manner resembling that seen in myotilinopathy patients. This is supported by the fact that the amount of myotilin in the patient's muscle samples is increased (Barrachina et al., 2007, Shalaby et al., 2009). Based on the results, we propose a model on the pathogenic mechanism, by which myotilin mutations induce muscular dystrophy. In this model, mutated myotilin is more resistant to degradation, which leads to accumulation of myotilin and induction of actin-containing protein aggregates. The aggregates may become toxic when sequestering essential cellular proteins and may eventually destroy the myofibers. This disease mechanism needs, however, further investigation in muscle cells. When Conover et al. (2009) transfected cardiomyocytes with mutated desmin, a protein found in MFM protein aggregates together with myotilin, endogenous desmin often colocalized with aggregates of the mutated form. In addition, we did not test all of the known myotilinopathy mutations and there might be several molecular mechanisms to cause the disease that starts pathologically with disintegration of the Z-disk (Selcen et al., 2004). There are still many open questions. Is

myotilin ubiquitinylated by murf-1 or other ubiquitin ligases? What hinders wt myotilin from aggregating in the cells? Desmin, for example, is prevented from aggregating under various forms of stress by the small heat shock protein alpha-B-crystallin (Perng et al., 1999). What is clear from these studies is that the cellular defects that lead to myotilinopathy are complex and that the mechanism of myopathy may involve alterations at many levels, including post-translational modifications and protein turnover.

It is only under continuous muscle contraction when it becomes apparent that the delicate balance between the expression and localization of different sarcomeric proteins is crucial for long-term maintenance; an observation that is supported by the data on mutated myotilin in humans and resulting different forms of muscle diseases. When there is more information about the interplay of sarcomere assembly and disassembly by proteolytic systems, the mechanisms of those muscle diseases where the balance of myofibril turnover is perturbed will become clearer. It will be important to gain greater understanding of both wt and mutated myotilin function through using tissue culture and animal models. The ultimate aim is to understand more about muscle structure and function to be able to develop effective treatments for muscle diseases.

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